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RNAi Targeting of Manduca sexta Using Chloroplast-Encoded Long dsRNA

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Abstract

Pest management strategies are crucial for reducing insect crop damage, and methods with species-specific effect are particularly desirable. Expressing dsRNA with sequence complementarity to a vital gene of feeding pests is a strategy that has shown potential. I modified the chloroplast genome of cultivated tobacco, *Nicotiana tabacum*, by adding a sequence encoding a 2222 base pair dsRNA complementary to the vacuolar-type H^+-ATPase subunit A mRNA of *Manduca sexta* and demonstrated expression of the product in leaves of the plants. Although feeding this same dsRNA that was synthesized *in vitro* to larvae knocked down expression of the target gene and induced lethality, chloroplast-encoded dsRNA had no effect on survival or target gene expression of feeding larvae. The results of this study suggest that a possible limitation for this strategy is the level of expression due to the length of the product, or that this strategy is inefficient for certain insect species.

Keywords: RNA interference, insecticide, *Manduca sexta*, pest management, agriculture, transplastomic, *Nicotiana tabacum*

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List of Abbreviations

aadA	Aminoglycoside adenyl transferase
Ago2	Argonaute-2
PCR	Polymerase chain reaction
Bla	Beta-lactamase
bp	Base pair
cDNA	Complementary DNA
CPB	Colorado potato beetle
CTAB	Cetyl trimethylammonium bromide
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EDTA	Ethylenediaminetetraacetic acid
EF1	Elongation factor 1
EF1α	Elongation factor 1-alpha
GFP	Green fluorescent protein
hpRNA	Hairpin RNA
LacZ	Beta galactosidase
LD_{50}	Lethal dose required to kill 50% of test subjects
MAPK	Mitogen-activated protein kinase
miRNA	Micro RNA
MS	Murashige and Skoog
nt	Nucleotide
NTC	No template control

PCR	Polymerase chain reaction
PVP	Polyvinylpyrrolidone
qRT-PCR	Quantitative reverse transcription PCR
Rep	Replication intiation protein
RISC	RNAi-induced silencing complex
RNA	Ribonucleic acid
RPM	Revolutions per minute
rps12	Ribosomal protein S12
rRNA	Ribosomal RNA
RTC	Reverse transcription control
RT-PCR	Reverse transcription PCR
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SID-1	Systemic RNA interference defective protein 1
siRNA	Small interfering RNA
SSC	Saline-sodium citrate
TE	Tris-EDTA
tkRNAi	Trans-kingdom RNAi
UTR	Untranslated region
v/v	Volume/volume
V-ATPase	Vacuolar-type H^+-ATPase
w/v	Weight/volume

Chapter 1: Introduction

Agriculture has been a cornerstone of society for nearly the entire history of civilization. Humans have been intentionally planting and harvesting crops since as early as 9400 B.C. (Kislev *et al.*, 2006), and in modern times agriculture is responsible for a sizable portion of the worldwide GDP; in Canada alone, this industry generates $100 billion per year (AAFC, 2018). Despite the crucial importance of agriculture, the current pest management strategies being used are not enough to prevent significant reduction in yield in response to herbivory and competition. Oerke and Dehne (2004) estimated the reduction in yield due to pests including weeds, insects, and pathogens to be as high as 40% on food crops such as rice and maize. While this loss is significant, loss of up to 80% of yield is estimated in the absence of pest management strategies (Oerke and Dehne, 2004).

Most of agricultural history has been marked by the use of chemical insecticides, ranging from natural resources like coal (Panagiotakopuli *et al.*, 1995) to more effective synthetic chemicals such as neonicotinoids (Schroeder and Flattum, 1984). Modern critical analysis of these approaches suggests health risks are associated with chemical exposure by humans and non-target organisms in the ecosystem (Hamblin, 2015). In addition, insects have reportedly developed resistances to a number of harmful chemicals, especially in response to excessive use (Cahill *et al.*, 2009). The RNA interference (RNAi) pathway has been proposed as a target for developing targeted, insect-specific insecticides. Feeding insects with double-stranded RNA (dsRNA) with sequence complementarity to mRNA encoding vital genes has been shown to successfully kill certain insects, and this strategy can be species-specific depending on the targeted gene and region within the mRNA (Whyard *et al.*, 2009).

The advent of technology allowing for the creation of genetically enhanced plants suggests a possible avenue for the development of sophisticated, pest-resistant crops. While nuclear transformation is the standard for plant genomics, studies which expressed dsRNA in the nucleus have been ineffective at knocking down gene expression in feeding insects, and there are no reported instances of highly lethal plants expressing nuclear

dsRNA. The observation that knocking down components of the RNAi pathway within the plant cells enhances insecticidal effectiveness suggests that dsRNA processing within the plant prevents sufficient silencing in feeding insects from taking place (Kumar *et al.*, 2012). Recent studies have proposed chloroplast transformation as a solution to this issue (Jin *et al.*, 2015; Zhang *et al.*, 2015).

In this study, I tested transplastomic tobacco, *Nicotiana tabacum*, as a delivery platform for insecticidal dsRNA targeting the tobacco hornworm, *Manduca sexta*, a popular lepidopteran model that is considered generally non-responsive to RNAi, especially through feeding. Notably, I tested a long (2222 base pair (bp)) dsRNA product, the first reported instance of a dsRNA molecule of this size being expressed within chloroplast. Testing this newly proposed insecticidal strategy with a variety of plants, transcripts, targets, and insects is crucial to characterizing its versatility and the extent of its effectiveness.

1.1. Agricultural Insecticides

1.1.1. Synthetic chemical insecticide and pest resistance

Strategies for reducing insect populations in agriculture have been utilized since as early as 1600 B.C. when natural substances such as coal and oil were used as means of warding pests away from crops (Panagiotakopulu *et al.*, 1995). In the 1900s, chemical pesticides based on arsenic and later the organochloride DDT found widespread usage, but with time the carcinogenic properties of these chemicals were discovered, amongst other negative human health effects, leading to an eventual ban from use in the United States (Bencko and Foong, 2017; Hamblin, 2015). Organophosphates, which function as inhibitors of insect cholinesterase, were frequently used over this period, but concerns regarding this family of insecticide eventually arose; for example, it was found that these chemicals result in over 300,000 deaths worldwide per year (Chatterjee and Riaz, 2013), and recently instances of pest resistance to these chemicals have been observed (Buzzetti *et al.*, 2016). Neonicotinoids are a popular alternative due to their insect-specific effects, but in recent years concerns over their role in declining honeybee, *Apis mellifera*, populations were raised leading to bans in regions such as the European Union (Wood

and Goulson, 2017). The worldwide cost of purchasing chemicals for field application is significant at nearly $40 billion per year (Pimentel, 2005). Over time, instances of insect populations developing resistance to common chemical insecticide methods have been reported, including incidences of multiple immunities by insects such as the Colorado potato beetle (CPB), *Leptinotarsa decemlineata,* (Alyokhin *et al.*, 2008) and diamondback moth, *Plutella xylostella,* (Santos *et al.,* 2011), suggesting a need for the development of flexible insecticide methods that can be altered in response to resistance.

1.1.2. Biological insecticides and genetically enhanced crops

Biological insecticides have been utilized as a means of avoiding the risks and financial costs associated with synthesizing and administering chemical-based insecticides in agriculture. The discovery of the insecticidal properties of the Gram-positive bacterium *Bacillus thuringiensis* was a major step toward the eventual development of pest control methods that are specific for particular pest insect species. *Bacillus thuringiensis* (Bt) express toxic, crystal-forming proteins called δ-endotoxins, which become denatured and cleaved within the alkaline environment of a feeding insect's midgut and insert into the lining of the midgut, preventing digestion from taking place (Pardo-López *et al.*, 2012). These proteins have lethal effects specifically toward insect species within orders Lepidoptera, Coleoptera, Hymenoptera, and Diptera (Schnepf *et al.,* 1998). In an effort to create insecticidal plants, the bacterial genes expressing Bt Cry toxins were introduced into tobacco through nuclear genome modification (Vaeck *et al.,* 1987), and the insecticidal effect of these plants was later improved through codon optimization allowing for higher expression of the toxin (Perlak *et al,* 1991). Genetically modified Bt cotton and Bt corn have been successfully commercialized. Subsequent research indicates that the economic benefits associated with such transgenic crops generally outweigh any potential risks (Shelton *et al.*, 2002), but concerns have arisen over the long-term effectiveness of this technology as insect populations will likely develop resistance with time. For example, evolution of resistance to Cry toxins by pink bollworm, *Pectinophora gossypiella,* in India via mutation of a cadherin receptor which recognizes the toxin has been observed, indicating a possible method by which insects worldwide could eventually develop resistance (Fabrick, 2010). Non-target effects of Bt crops on insect populations

have been investigated and continue to be frequently researched, although thus far the effects on species of concern such as honeybee (Duan *et al.*, 2008) and Monarch butterfly, *Danaus plexippus* (Sears *et al.*, 2001) seem to be negligible. Another concern regarding this strategy is that genes encoding toxins in Bt crops are dominant, and the possibility of insect resistance emerging in wild type populations due to crossover has been proposed (Brévault *et al.*, 2015). Multiple studies have detected the presence of Bt transgenes in wild type maize populations in Mexico (Piñeyro-Nelson *et al*¸ 2008; Quist and Chapela, 2001), although the methodology and results of these studies have been called into question (Schoel and Fagan, 2009).

1.2. RNA Interference

1.2.1. History and overview of RNAi

The biological pathway that would later be described as RNA interference was first reported when an attempt to overexpress a gene associated with petal pigmentation in petunia plants unexpectedly resulted in gene suppression (Napoli *et al.*, 1990). The first documented instance of RNAi in an animal species involved the nematode *Caenorhabditis elegans* wherein injection into oocytes of a plasmid expressing antisense RNA resulted in an observable phenotypical change associated with downregulation of the complementary gene; it was later discovered in the same species that double-stranded RNA was far more effective at producing the downregulation effect compared to single-stranded (Fire *et al.*, 1991; Fire *et al.*, 1998). RNAi subsequently became a useful tool in a wide variety of biological studies with applications ranging from elucidation of gene function to possible therapeutic use in humans.

There are two broadly described RNAi pathways which differ primarily in the origin of the dsRNA. The endogenous pathway has been observed in fungi, plants, and animals, as a means of regulating gene expression through expression of approximately 25 nucleotide (nt) long micro RNAs (miRNAs) which are upregulated in response to stimuli. For example, the downregulation of transcription factors in response to external stress in plants is affected through this pathway (Sharma *et al.*, 2015). The exogenous pathway, on

the other hand, occurs when dsRNA originates externally and is taken up by the cell within which it will enter the RNAi pathway. This includes instances of trans-kingdom RNAi (tkRNAi), wherein dsRNA is expressed by an organism within one Kingdom and transferred to another. Examples of tkRNAi include transfer from bacteria to animals (Xiang *et al.*, 2006) and from fungi to plants (Wang *et al.*, 2016).

1.2.2. Mechanism of RNAi

With exogenous RNAi, intact exogenous dsRNA is detected by the protein Dicer which cleaves the substrate into small interfering RNAs (siRNAs), which are approximately 22 nt in length (Bernstein *et al.*, 2001). The mechanisms of the exogenous and endogenous RNAi pathways proceed similarly beyond this step, with any variation in effect attributed solely to the sequence of the dsRNA (Hutvágner and Zamore, 2002). siRNAs or miRNAs are integrated into the RNA-induced silencing complex (RISC) which is made up of multiple proteins with various functions. Only a single strand is selected by RISC via a sequence-specific method. Typically, whichever strand has a less stable 5’ end according to base pairing rules, meaning a lower GC-content, is selected (Schwarz *et al.*, 2003). The opposite strand, called the passenger strand, is cleaved and degraded (Leuschner *et al.*, 2006). The loaded RISC complex subsequently binds the target mRNA and guides the single-stranded siRNA into tagging its complementary mRNA via Watson-Crick base pairing. Here, the target differs between miRNA and siRNA with the former typically binding the 3’ untranslated region (UTR) and the latter binding the translated region, although siRNA has been shown to successfully tag UTRs as well (Doench *et al.*, 2003). If perfect complementarity is present, tagged mRNA is cleaved by the argonaute-2 (Ago2) protein domain of RISC, thus preventing translation (Liu *et al.*, 2004). Imperfect complementarity, usually associated with miRNA binding to 5’ UTRs, prevents expression by inhibiting the translational machinery rather than degradation of the mRNA (Saxena *et al.*, 2003). The exogenous RNAi pathway is illustrated in Figure 1.1.

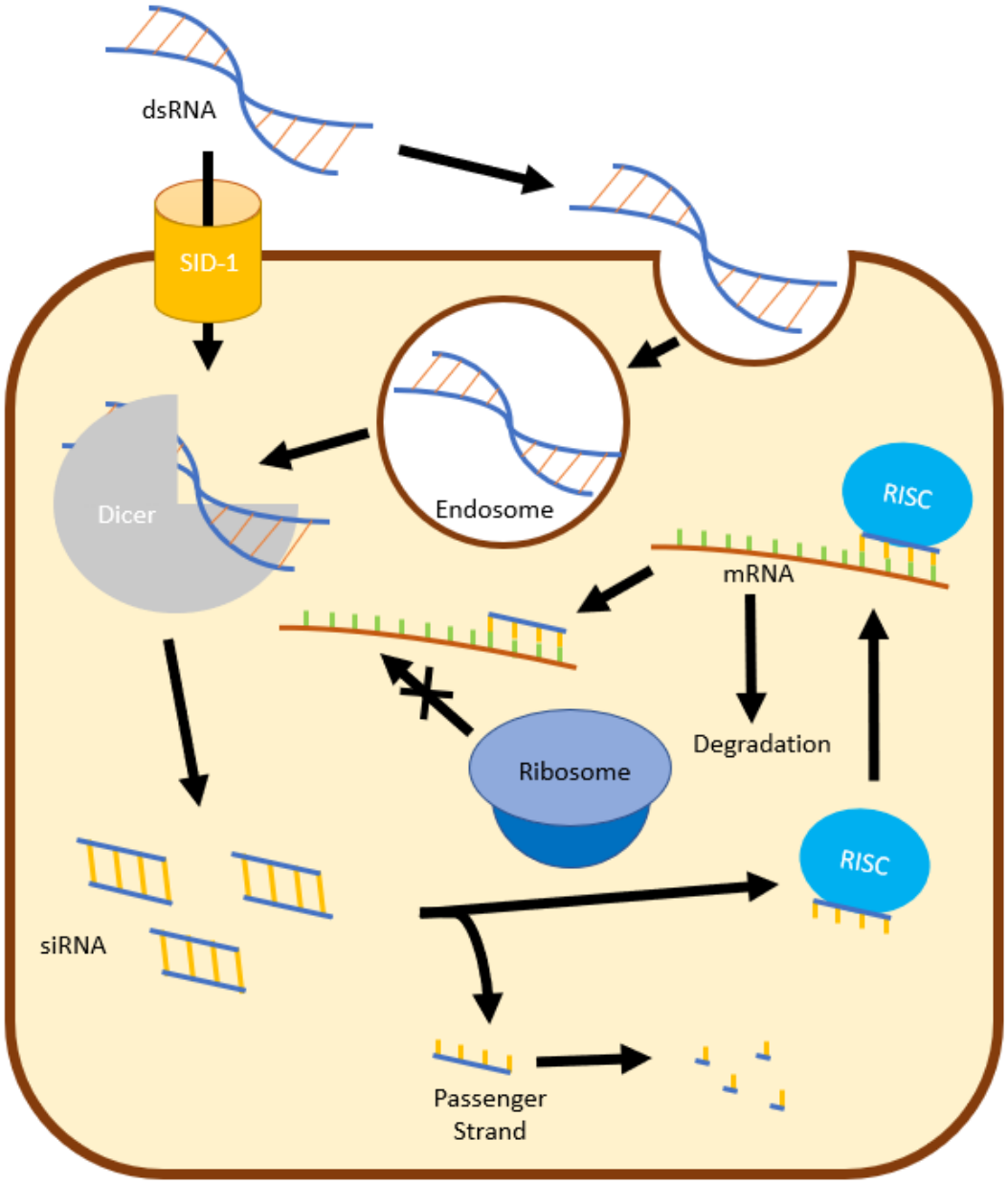

Figure 1.1. Uptake and RNAi pathway of exogenous dsRNA in an insect cell. dsRNA is taken up by insect cells through passive SID-1 channels or through endocytosis. Once internalized, Dicer cleaves dsRNA into siRNA. siRNA is assembled into the RISC, during which the passenger strand is released and degraded. The guide strand is used to recognize its complementary mRNA, which is subsequently degraded or prevented from translation depending on the degree of complementarity.

1.2.3. RNAi in insect species

The class Insecta contains a highly diverse range of species found in nearly every square kilometer of land on the planet Earth and includes more than half of all described organisms and a sizable portion of all species within the kingdom Animalia; the crucial importance of this clade to human life is thus well-described. Biological tools allowing for the elucidation of insect gene function or to modify insect behaviour for various purposes are of great interest for entomological research. The use of RNAi as a means of knocking down insect gene expression has been researched extensively since the discovery of the pathway as described previously.

The RNAi system can be used to silence expression of single genes, allowing examination of the consequence to the resulting phenotype and therefore deducing the functions of the products of individual targeted genes. Initial studies to investigate gene function using RNAi in insects were performed *in vivo* using *Drosophila melanogaster*. In this model, the insertion of transgenes expressing hairpin RNAs (hpRNAs), long reverse-complementary RNAs that base-pair with themselves, was performed to generate libraries of transgenic lines featuring single gene knockouts activated conditionally via GAL4/UAS expression (Dietzl *et al.*, 2007). While the *in vivo* method does provide insight into the function of a wide range of genes expressed by *D. melanogaster*, it falls short when considering gene function in non-model organisms for which targeted gene expression is less well-described. Multiple approaches have been utilized for inducing RNAi in non-Drosophilid insects with success varying between, and occasionally within, species. Injection of dsRNA synthesized *in vitro* into insect haemolymph has been successfully used to knock down gene expression in species within the orders Coleoptera (Tomoyasu and Denell, 2004), Diptera (Sanscrainte *et al*., 2018), and Orthoptera (Ren *et al.*, 2013), amongst others. Soaking in dsRNA was sufficient for knocking down gene expression within hemipteran larvae (Yu *et al.*, 2017) and within insect cell cultures derived from a variety of species (Yu *et al.*, 2013).

Oral delivery of dsRNA is a favourable method due to the passive nature of uptake in contrast with injection, but this approach adds the additional variable of exposure to the

contents of the midgut. Feeding is especially successful in species within the order Coleoptera, although generally with lower effectiveness compared to injection, a comparison which was demonstrated through application to the sweet potato weevil, *Cylas puncticollis* (Prentice *et al.*, 2016). In species within the order Hemiptera, effectiveness of orally-induced RNAi varies within species. Oral delivery of dsRNA was successful at knocking down gene expression in potato psyllid, *Bactericerca cockerilli*, (Wuriyanghan *et al.*¸ 2011) but not in tarnished potato bug, *Lygus lineolaris*, (Allen and Walker, 2012), and with varying levels of success in pea aphid, *Acyrthosiphon pisum* (Christiaens *et al.*, 2014; Whyard *et al.*, 2009). This last example indicates possible intra-strain variation in RNAi response. In Hemiptera, the failure of ingested dsRNA to knock down gene expression seems to be due to enzymatic activity within the saliva of the feeding insect, as exposure of dsRNA to saliva *in vitro* results in rapid degradation (Allen and Walker, 2012). In some organisms, dsRNA and thus the effects of RNAi can spread from cell to cell, as was first observed in an animal model using *C. elegans* (Hunter *et al.*, 2006). This has also been reported in some insect species, and usually correlates with effectiveness; for example, a systemic RNAi response occurs in red flour beetle, *Tribolium castaneum*, a species within the highly RNAi-sensitive order Coleoptera (Tomoyasu *et al.*, 2008).

1.2.4. RNAi in lepidopteran species

Of interest to researchers is the potential for use of RNAi to knock down gene expression in species within the order Lepidoptera, a clade which consists of moths and butterflies. Many adult lepidopterans are considered valuable pollinators, while many larvae are characterized as agricultural pests due to their abundant feeding upon crops. Low efficiency of RNAi is a common issue in genetic research regarding lepidopterans. Successful attempts at inducing RNAi through the three methods described previously – soaking (Wang *et al.*, 2011), injecting (Quan *et al*., 2002), and feeding (Turner *et al.*, 2006) – have been reported in lepidopteran species, but the success of any of these approaches is highly species-dependant. Similar to the saliva of hemipterans, the lack of RNAi response in species of lepidopterans seems to be attributable to the presence of dsRNA degrading enzymes within the midgut environment of the insect. Wang *et al.*

(2016) reported no RNAi response through feeding tobacco cutworm, *Spodoptera litura*, with dsRNA compared to a significant downregulation response in American cockroach, *Periplaneta Americana*, and Giant mealworm beetle, *Zophobas atratus*, a result which correlated with the observation of significant degradation of dsRNA after incubation in extracted midgut contents of the foremost but not the latter two species. Heating midgut contents or adding a chelating agent such as ethylenediaminetetraacetic acid (EDTA) reduces the dsRNA degradation ability, which suggested a metal-dependant enzymatic origin for this property (Garbutt *et al.*, 2013). Guan *et al.* (2018) identified a lepidopteran-specific protein with structural similarity to other known nucleases and observed that expression is upregulated in response to application of dsRNA. It has been theorized that this protein functions to assist the insect in digesting nucleic acids consumed from plants for sequestering and use in biosynthetic pathways (Arimatsu, *et al.*, 2007). Interestingly, there appears to be another barrier preventing efficient RNAi in lepidopterans that occurs beyond cellular uptake. Shukla *et al.* (2016) observed that tobacco budworm, *Heliothos virescens* cells in culture are capable of taking up dsRNA at the same rate as cells from CPB, but that processing into siRNA occurred far more inefficiently in the former species, and suggest that internalization into and accumulation within endosomes is the limiting factor. Such accumulation of dsRNA within endosomes in *S. litura* was observed by Yoon *et al.* (2017).

1.2.5. Mechanism of dsRNA uptake in insect midgut cells

Two methods by which dsRNA is taken up by insect midgut cells have been identified. The first method requires the presence of selective RNA interference defective protein (SID-1), a dsRNA-specific passive transmembrane channel first identified as essential for systemic RNA in *C. elegans* and later revealed as capable of sequestering dsRNA from the environment when expressed via plasmid transfection into *D. melanogaster* cells (Winston *et al.*, 2002; Feinberg and Hunter, 2003; Shih and Hunter, 2011). SID-1 orthologues have been identified in insects within the orders Coleoptera, Hymenoptera, Hemiptera, Lepidoptera, and Orthoptera, amongst others (Tomoyasu *et al.*, 2008; Aronstein *et al.*, 2006; Xu and Han, 2008; Dong and Freidrich, 2005; Huvenne and Smaghe, 2010). Tomoyasu *et al.* (2008) reported the presence of three *sid-1*-like genes in

a lepidopteran species, the silkworm *Bombyx mori*. Facilitation of dsRNA uptake from the environment in *C. elegans* also depends on the presence of an accessory protein, SID-2, which is specifically expressed within gut cells (Winston *et al.*, 2007), but no orthologues for this protein have been observed in insects (Cappelle *et al.*, 2016).

The observation that dipteran species such as *D. melanogaster* and yellow fever mosquito, *Aedes aegypti*, do not express SID-1-like proteins but are still responsive to RNAi via external application suggests the presence of a second method for dsRNA uptake by insect cells (Tomoyasu *et al.*, 2008). Ulvila *et al.* (2006) performed a screen to identify genes in *Drosophila* upon which dsRNA uptake was dependent and discovered that knockdown of proteins associated with endocytosis greatly impaired RNAi, and specifically identified two scavenger proteins, SR-CI and Eater, which are responsible for 90% of dsRNA uptake by *Drosophila* S2 cell cultures. Cappelle *et al.* (2016) observed the endocytosis-mediated pathway in CPB midgut which suggests that this pathway could be conserved in a variety of insect orders. Internalization of dsRNA into insect cells through endocytosis and SID-1 mediated uptake are illustrated in Figure 1.1.

1.2.6 Effect of dsRNA size on RNAi efficiency

Length of the dsRNA product used to induce RNAi has been identified as a factor affecting success in various systems. Generally, longer products have been associated with greater effectiveness in insects, as was observed through comparison between a 480 bp product and <100 bp products in *T. castaneum* (Miller *et al.*, 2012). The stage of dsRNA uptake and utilization by the insect RNAi pathway at which a longer product is more effective is not fully understood. Shih *et al.* (2009) suggested that the difference does not occur at the stage of cellular uptake as long and short dsRNA accumulated within *Drosophila* S2 cells at similar levels despite the former product inducing RNAi to a greater degree. However, differences between dsRNA uptake between Diptera and other insects could suggest that this explanation is not applicable for all insects.

1.3. RNAi-Based Insecticide Methods

1.3.1. *In vitro* synthesized insecticidal dsRNA

The concerns over off-target effects of traditional biological and synthetic chemical insecticide methods have indicated a need for alternatives that can be designed to target only single undesirable species. dsRNA could therefore make for an ideal insecticidal product due to its ability to knock down gene expression in a manner that can be designed to be specific at the species level, and its inability to affect mammals in any measurable way due to biological barriers within the gut, even at extremely high doses (Petrick *et al.*, 2015). As proof of the species-specificity capabilities of RNAi, Whyard *et al.* (2009) determined that targeting a highly variable region of a gene, specifically the 3' UTR of a vacuolar-type H^+ -ATPase (V-ATPase) transcript, was sufficient for inducing a lethal response that differed between closely related species within the genus *Drosophila.*

Use of dsRNA as an insecticide relies on application methods that can be implemented in a field or greenhouse setting, narrowing the delivery methods to topical exposure or, more likely, oral delivery via feeding. While not yet applied in a commercial setting, proof of concept investigations into the use of insecticidal dsRNA have been conducted. Application of CPB specific dsRNA to potato leaves in a greenhouse setting resulted in stunted growth and mortality of feeding insects over a 28-day period with dsRNA remaining present and stable throughout the duration of the experiment, even withstanding washes with water (Miguel and Scott, 2015). Another insecticidal application of dsRNA is to knock down expression of genes related to insecticide resistance, so that the efficiency of an insecticide applied in conjunction with the dsRNA is greater than when the insecticide is applied on its own. This innovative strategy was successfully applied through knockdown of cytochrome P450 genes in combination with imidacloprid application in Asian citrus psyllid, *Diaphorina citri* (Killiny *et al.*, 2014).

The wide variety in feeding mechanisms employed by insects presents an issue for this application of insecticidal dsRNA. For example, species within order Hemiptera, which includes common agricultural pests such as whitefly and the brown marmorated stink

bug, generally feed via sap-sucking. No strategy for practical lethal application of dsRNA for ingestion by these insects has been successful, although root uptake in a hydroponic setting has been suggested (Ghosh *et al.*, 2018). For lepidopterans, exposure to insecticidal dsRNA synthesized *in vitro* is generally ineffective for use in practical setting: as discussed previously, dsRNA is degraded within the harsh midgut environment of these insects, rendering it unable to knock down gene function to lethal levels.

1.3.2. Nuclear-encoded insecticidal dsRNA

Bioencapsulation through expression within plant cells has been proposed as a delivery method for ingested dsRNA in insects that are generally resistant to *in vitro* synthesized dsRNA. This was first achieved through insertion of dsRNA-encoding sequences into the nuclear genome of corn, *Zea mays*, for successful knockdown of vital gene expression in feeding western corn rootworm, *Diabrotica virgifera*, (Baum *et al.*, 2007) and into the nuclear genome of *N. tabacum* and *Arabidopsis thaliana* for knockdown of gene expression of detoxication enzymes in feeding cotton bollworm, *Helicoverpa armigera*, larvae (Mao *et al.*, 2007). Although significant knockdown has been achieved in studies such as these, there are few, if any, reports of fully insect-resistant plants through insertion of dsRNA-encoding sequences into a plant nuclear genome. This is certainly true regarding lepidopteran targets, wherein successful studies generally knock down gene expression to increase susceptibility in conjunction with an applied chemical insecticide, such as the above study in *H. armigera* that decreased pest resistance to gossypol (Mao *et al.*, 2007). While it is possible for this approach to play a role in an insecticide strategy, the application of chemicals is not ideal, and development of alternate resistance strategies is still a risk. Plants, like other eukaryotes, have an innate RNAi pathway and thus express homologs to proteins such as Dicer (Bernstein *et al.*, 2001). Mao *et al.* (2007) hypothesized that the low level of gene knockdown by ingested dsRNA expressed *in planta* is likely due to processing by components of the RNAi pathway within the cytoplasm of the plant cells, a hypothesis which they supported by developing Dicer knockout plant lines also expressing dsRNA which knocked down insect gene expression with much greater success.

1.3.3. Chloroplast-encoded insecticidal dsRNA

The observation that knockdown of gene expression in feeding insects is far more efficient using plants expressing intact dsRNA invited speculation into methods that could protect the insecticidal product from cleavage by Dicer in plant cells. Feeding of dsRNA encapsulated within bacteria is a method of delivery that has been successful (Tian *et al*, 2009), indicating that dsRNA remains stable within the confines of a prokaryotic cell. This observation suggests that chloroplasts, which evolved from free-living cyanobacteria forming an endosymbiotic relationship with eukaryotic cells, could provide a similar level of protection for dsRNA from degradation. Zhang *et al.* (2015) tested this hypothesis through transplastomic genome modification of potato plants to induce expression of dsRNA targeting β-actin and Shrub proteins of CPB, which successfully induced lethality in these insects upon feeding. Similar results were observed in two other studies using genetic modification of tobacco chloroplast with dsRNA-encoding sequences targeting gene expression in *H. armigera* (Jin *et al*., 2015; Bally *et al.*, 2016). Zhang *et al.* (2015) confirmed an abundance of intact plastid dsRNA compared to nuclear expression of the same dsRNA using Northern blot analysis, as the latter strategy resulted in a large amount of processed siRNA compared to the former. These three successful examples of plastid-expressed dsRNA differed in the method by which dsRNA was expressed. Zhang *et al.* (2015) utilized plasmid constructs expressing dsRNA via two flanking convergent RNA polymerase promoters for their bioassays after determining that this method expressed the highest quantity of intact dsRNA compared to methods using a hairpin structure. Jin *et al.* (2015) utilized hpRNA expressed via a single sequence promoting transcription of reverse complementary sequences joined together by a 200 bp hairpin loop. Bally *et al.* (2016) improved upon this method by reducing the length of the hairpin to 21 bp, which successfully reduced the amount of post-transcriptional processing of the product. Figure 1.2 summarizes the differences between the chloroplast and nuclear dsRNA expression strategies including the difference in the end products of these two pathways.

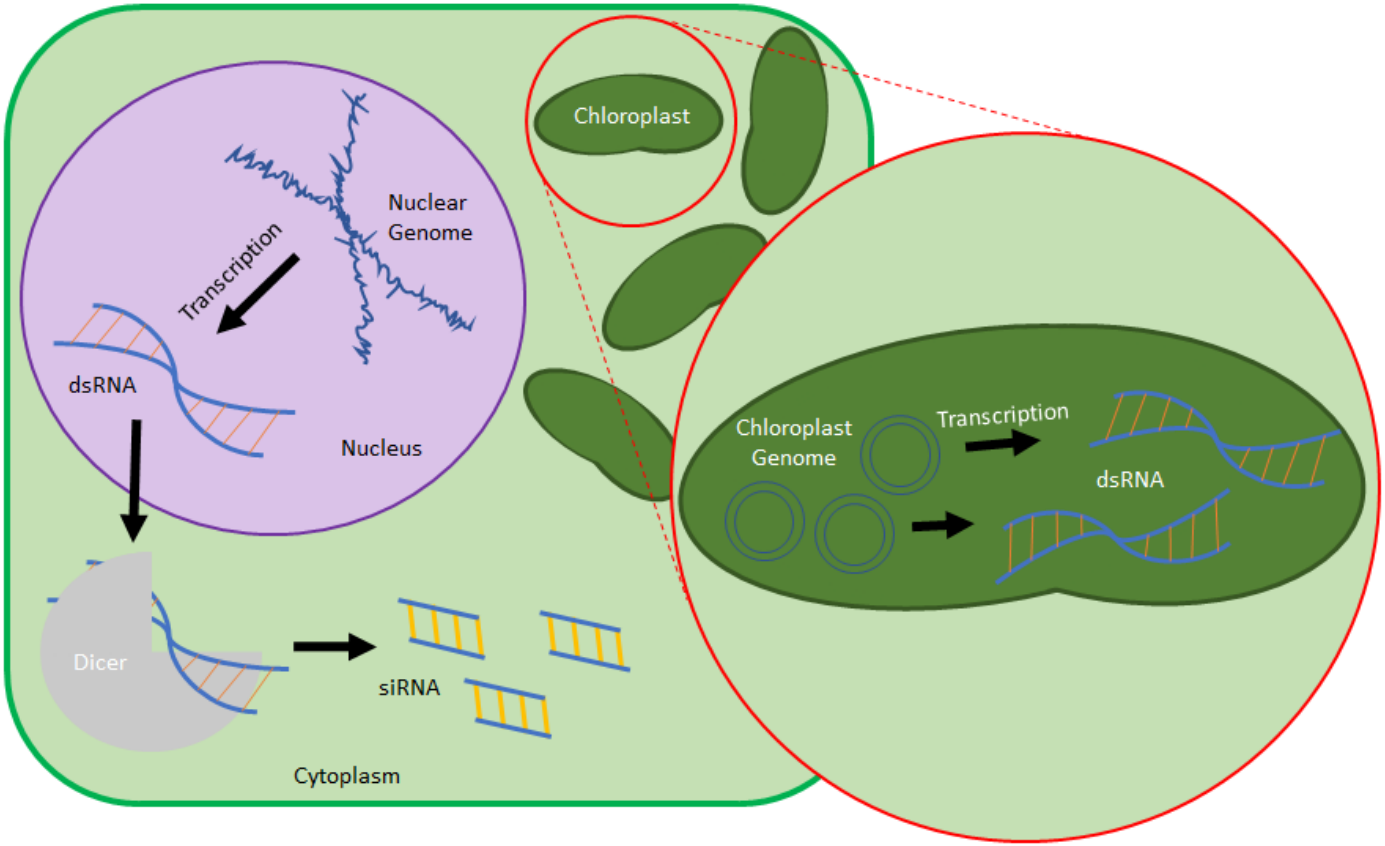

Figure 1.2. Comparison of nuclear and chloroplast dsRNA expression methods in a plant cell. Genetically modified nuclear DNA contains a single copy of the dsRNA sequence per cell. dsRNA is transcribed and transported into the cytoplasm, where the plants endogenous Dicer proteins cleave it into siRNA. Genetically modified chloroplast DNA contains multiple sequences in a single cell due to the high copy number of the chloroplast genome in each chloroplast as well as high number of chloroplasts per cell. dsRNA is transcribed and stored intact within the stroma of the chloroplast.

1.4. Tobacco *Nicotiana tabacum*

1.4.1. Taxonomy and description

N. tabacum (Solanaceae: Solanales), commonly known as cultivated tobacco, is a flowering annual plant within the nightshade family, a clade which includes common agricultural crops such as tomatoes, potatoes, peppers, and eggplant. Native to Central and South America, cultivated tobacco is now grown worldwide. Genetic analysis indicates that *N. tabacum* arose through crossover events between three other species of tobacco: *N. sylvestris, N. otophora, and N. tomentosiformis* (Ren *et al.*, 2001). *N. tabacum* plants can grow up to 2 metres within a year while producing an abundance of leaf tissue and seeds. Tobacco is used as a model organism in numerous studies regarding plant genetics and biology, for example through the development of reference genomes and transcriptomes (Sierro *et al.*, 2013).

1.4.2. Agricultural and commercial use of tobacco

Tobacco is commonly grown for human consumption due to its production of the stimulant nicotine, which constitutes up to 3.6% of total plant mass in some cultivars (Hossain and Salehuddin, 2013). Nearly one-fifth of the global population uses tobacco on a regular basis (Esson and Leeder, 2004) and over 7 million tonnes of tobacco plant tissue are consumed each year (FAO, 2003). One proposed use for tobacco outside of its commercial role would be as a trap crop to be grown alongside agriculturally important crops. For this, the tobacco genome could be modified to express compounds that attract pests as well as compounds that kill the pests upon feeding. This approach is particularly feasible in tobacco due to the vast wealth of genetics knowledge amassed for this species. Genetic modifications to enhance the attractiveness of tobacco to certain insect species have already been successful. For example, the genetic pathway which produces the compound benzylglucosinolate in *A. thaliana* has been transformed into the tobacco nuclear genome, which successfully increased diamondback moth, *Plutella xylostella*, oviposition rates in comparison to wild type (Møldrup *et al.*, 2011). Another commercial

use of tobacco is through extraction of nicotine, which can be dusted onto crops as a natural defense from pests in a field setting (Casanova *et al.*, 2002).

1.4.3. Chloroplast genome transformation of tobacco

Chloroplast genome transformation of *N. tabacum* is well-described due to favourable properties of this plant including fast growth, robust regeneration on tissue culture (Horsch *et al.*, 1985), and availability of the entire chloroplast genome sequence (Gao *et al.*, 2016). Transient chloroplast transformation was initially reported through the insertion of a plasmid encoding chloramphenicol acetyltransferase into tobacco cells (Daniell *et al.*, 1990). Svab *et al.* (1990) successfully generated stable expression of an antibiotic resistance marker in tobacco through biolistic particle delivery using tungsten particles coated in plasmid encoding a modified 16S ribosomal RNA (rRNA) sequence conferring spectinomycin resistance. The modified sequences were transferred to the chloroplast genome via homologous recombination. All seed progeny exhibited the resistance phenotype, which suggested that the transgene was inheritable and likely homoplastomic. This strategy was later improved on by using two flanking homologous recombination sites surrounding an expression cassette containing a gene encoding aminoglycoside resistance protein, which degrades spectinomycin (Svab and Maliga, 1993).

There are several benefits attributed to chloroplast transformation in tobacco which make it a favourable alternative to nuclear transformation. Staub *et al.* (2000) reported that tobacco chloroplasts are inherited maternally due to the observation that all transplastomic female crosses with wild type males resulted in transplastomic progeny, but not vice-versa. This observation is consistent in many species of plants (Fauré *et al.*, 1994; Gastony *et al.*, 1992). This property is ideal for developing transgenic plants for use in a field setting as the previously discussed risk of crossover into wild type populations is reduced. Another benefit of chloroplast transformation via biolistic particle delivery is that insertion is site-directed according to homologous recombination sites which can be identified using the available tobacco chloroplast genome. This eliminates the chance of position effects occurring from random insertion, an issue often attributed to nuclear

transformation methods such as agrobacterium-mediated transformation (Gelvin, 2003). Another advantage is the high copy number of the chloroplast genome within each chloroplast and the high number of chloroplasts within most plant tissue, which allows for a large amount of protein or nucleic acid production to take place following transformation. Oey *et al.* (2009) found that expression of a transplastomically produced protein in tobacco accounted for more than 70% of the plant's total soluble protein, and that expression could be upregulated enough to exhaust the capacity of the plastid to translate protein.

Chloroplast transformation of tobacco has been proposed as a method for low-cost, large-scale generation of a number of biological compounds for use in various industries. One application for transplastomic tobacco is for production of edible biopharmaceuticals. For example, the expression of antigens for tuberculosis (Lakshmi et al., 2013) and anthrax (Gorantala *et al.,* 2014) have been achieved. Transplastomic tobacco plants have also been proposed as a means of generating large volumes of insulin for oral delivery (Boyhan and Daniell, 2011). Biomaterials and industrial enzymes such as hydroxybenzoic acid (Madanala *et al.*, 2015) and endo-glucanase (Petersen and Bock, 2011) respectively have been successfully generated to significant levels in tobacco using the chloroplast.

1.5. Tobacco Hornworm *Manduca sexta*

1.5.1. Taxonomy, description and life cycle

Tobacco hornworms, *M. sexta* (Linnaeus) (Lepidoptera: Sphingidae) are large, hyperphagic larvae of the Carolina sphinx moth. Adult moths select non-damaged host plants and lay around 100-120 eggs per day, which are about 1 mm in diameter and emerald-green (Sasaki and Riddiford, 1984; Reisenman *et al.*, 2013). Oviposition and feeding occurs typically on plants within the order Solanaceae. Immediately after hatching, larvae consume their egg cases and then proceed feed upon leaf tissue (Reinecke *et al.*, 1980). Larvae select food sources through detection of compounds on the surface of their ideal solanaceous host plants (del Campo *et al.*, 2001) and through chemosensation of water vapour which leads them toward sources of hydration (Rowley

and Hanson, 2007). Under laboratory conditions, *M. sexta* larvae pass through five instars and pupate after about 23 days when fed on an ideal wheat germ artificial diet, growing from around 1 mg after hatching to over 9 g at their peak size (Reinecke *et al.*, 1980). Short-day conditions induce diapause in pupating insects, which can last around 97 days, compared to non-diapause pupation which lasts around 21 days (Reinecke *et al.*, 1980, Kingsolver 2007). Following pupal ecdysis, adult moths forage, mate, and lay eggs for about two weeks (Contreras *et al*., 2013). Larvae of *M. sexta* are a popular insect model for research studying lepidopteran physiology and genetics, and guidelines for mass rearing under laboratory conditions have been developed (Yamamoto, 1969).

1.5.2. Pest status

Manduca sexta larvae are typically considered garden pests but are also an occasional nuisance in agricultural settings. Larvae display feeding preference through the detection of a glycoside present on the leaves of certain solanaceous plants, and their typical targets include leaves from tomatoes and tobacco (del Campo *et al*., 2001). Larvae feed voraciously on tobacco leaves, with a single late-instar larva capable of consuming several large leaves in only a few days (Pavuk, 2009). Unlike most insects, *M. sexta* are resistant to the negative effects of consuming nicotine. Larvae are capable of sequestering and repurposing this alkaloid as a method of defense from predation, therefore allowing themselves protection from many natural enemies (Kumar *et al.*, 2014). There are a few approaches utilized today to reduce the damage from *M. sexta* infestation on crops. Late-instar larvae are easy to observe and remove mechanically, but are more resistant to toxins and harder to control through strategies such as *B. thuringiensis* application (Pavuk, 2009). One method of biological control that is used and effective against *M. sexta* is parasitization by the wasp species *Cotesia congregata* (Lavine and Beckage, 1996). More recently, vital metabolic pathways within *M. sexta* that are potential targets for disruption and induction of lethal effects have been identified (Li *et al.*, 2017). *M. sexta* is often used as a model for molecular biology research attempting to locate pathways and resistance mechanisms in other insect species, or to discover genes that can be upregulated or downregulated to increase insecticide susceptibility (Arenas *et al.*, 2010; Porta *et al.*, 2011).

1.5.3. Observations of RNAi in *M. sexta*

The response of *M. sexta* to RNAi is generally quite low, especially in comparison with insects belonging to orders such as Coleoptera or Blattodea. Injection of dsRNA directly into the haemolymph has been shown to successfully knock down gene expression in *M. sexta* (Eleftherianos *et al.*, 2006) but the effect is variable and dsRNA is not persistent in haemolymph plasma compared to more sensitive species like the German cockroach, *Blatella germanica* (Garbutt *et al.*, 2013). While there are some reports of successful dsRNA application through sustained feeding over time (Whyard *et al.*, 2009), a more common approach for inducing RNAi orally in *M. sexta* is by droplet feeding. This strategy, which generally requires starvation of the insect followed by administration of a 1 µL drop of highly concentrated dsRNA, has been used for various purposes in the study of *M. sexta* genetics. For example, this method has been used to measure the effect of mitogen-activated protein kinase (MAPK) p38, aminopeptidase-N, alkaline phosphatase, and cadherin knockdown on susceptibility to Cry toxins (Cancino-Rodezno *et al.*, 2010; Flores-Escobar *et al.*, 2013; Gómez *et al.*, 2015) and to observe the effects of knocking down expression of chemosensory genes on both larvae and adults (Howlett *et al.*, 2012). Another oral delivery method that has been utilized is transient expression of dsRNA products using tobacco rattle virus vector transfection of *Nicotiana attenuata*, which was efficient in combination with knockdown of the gene encoding Dicer (Kumar *et al.*, 2012).

1.5.4. Vacuolar-type H^+-ATPase

Many lepidopteran species feature a highly alkaline midgut environment, a trait which appears to have evolved as an adaptation to diets containing tannins and chlorogenic acid (Clark, 1999). pH levels as high as 11.2 have been observed in the midgut lumen of certain lepidopteran species, and *M. sexta* are no exception with an average midgut pH of 10.8±0.25 (Gringorten *et al.*, 1993). Consequently, enzymes within the midgut of lepidopterans such as digestive α-amylases (Pytelková *et al.*, 2009) and nucleases (Schernthaner *et al.*, 2002) have adapted to function efficiently at a high pH, and disruption of this pH can affect the insect's phenotype. Dow (1992) observed that the pH

gradient in *M. sexta* gut is generated through the mechanism of V-ATPase protein pumps expressed in the epithelium that sequester hydrogen ions from the lumen into the cytoplasm of the midgut cells. Whyard *et al.* (2009) knocked down gut expression of V-ATPase subunit E to lethal levels in *M. sexta*, which suggests that components of this pathway are an ideal target for insecticidal RNAi. The structure of the V-ATPase complex in *M. sexta* is made up of 12 subunits, and disruption of any of these components reduces function of the entire complex (Wieczorek *et al.*, 2009). In Western corn rootworm, knockdown of subunit A specifically had the greatest effect compared to subunits E and D (Baum *et al.*, 2007).

1.6. Objectives

With time, insect populations develop resistance and eventual immunity to the various insecticidal methods used to control their presence on crops. RNA interference is a promising tool for targeting insects in a species-specific way that can be adjusted in response to resistance through target modification, but innate physiological properties of some insect species create biological barriers that must be overcome in order for dsRNA delivery and gene knockdown to be successful. Strategies such as *in vitro* synthesized and nuclear-encoded dsRNA have thus far achieved only partial success, especially for species within the highly problematic order Lepidoptera. Chloroplast-encoded dsRNA is a possible solution, as bioencapsulation of the insecticidal product within the plastid could provide protection from cellular processing prior to insect uptake. The initial objective of this study was to determine the efficiency of dsRNA products targeting the V-ATPase subunit A mRNA at inducing RNAi in *M. sexta* through oral ingestion and any effects of this knockdown on insect survival. The second objective was to generate a stable, homoplastomic line of *N. tabacum* plants expressing this dsRNA using chloroplast genome modification. The final objective was to rear *M. sexta* larvae on leaves from transplastomic *N. tabacum* to observe lethality and knockdown of gene expression in comparison to the *in vitro* results.

Chapter 2: Materials and Methods

2.1. Tobacco Chloroplast Transformation

2.1.1. Tobacco Growth and Variety

The tobacco variety used for this study was the variety '81V9', a cultivar which produces low amounts of alkaloids (Menassa *et al.*, 2001). It was assumed that this variety would reduce the possibility of insect rejection in response to nicotine. Seeds were sterilized by a 5 min wash with 70% volume per volume (v/v) ethanol and germinated at room temperature and under a 16L:8D photoperiod in Magenta vessels (Sigma-Aldrich) (77 mm x 77 mm x 97 mm) containing 100 mL Murashige and Skoog (MS) medium (0.22% weight per volume (w/v) MS Salt mixture, 3% w/v sucrose, 0.05% w/v agar). Once plants grew to about 9 cm tall, they were transferred to pots containing soil for further growth. Plants on soil were fertilized once every two days with 0.05% w/v high nitrate fertilizer (20% nitrogen, 8% phosphoric acid, 20% potash). Tobacco plants were grown under ambient conditions in greenhouses or at room temperature (22ºC±1ºC) under a 16L:8D photoperiod in growth cabinets. Tobacco tissue culture experiments were performed at room temperature under a 16L:8D photoperiod.

2.1.2. Plasmid Design and Cloning

The plasmids utilized for tobacco chloroplast genome transformation were prepared prior to the beginning of this project. Empty pTom plasmids were designed by Dr. Igor Kolotilin (personal communication) (Fig. 2.1). This plasmid includes a gene encoding the enzyme β-lactamase (Bla), which confers antibiotic resistance through degradation and allows for selection of positive bacterial transformants in the presence of ampicillin. The expression cassette region of the plasmid is flanked by two segments of 1544 nt and 1513 nt, Flanx I and Flanx II respectively, with identical sequence to a region within the ribosomal protein S12 (*rps12*) encoding region of the tomato chloroplast genome, the sequence of which is sufficiently homologous to that of the tobacco chloroplast to allow for homologous recombination between the plasmid and the genome. The *rps12* region is

repeated twice within the chloroplast genome, meaning the transgene will be present in duplicate following homologous recombination within the chloroplast and subsequent selection pressure. The expression cassette contains a gene encoding aminoglycoside adenyl transferase (*aadA*), under the control of a psbA promoter that is active in chloroplasts. This protein adenylates spectinomycin, causing it to degrade, thus allowing use of this antibiotic for selection of transformants. The expression cassette also contains a region flanked by two constitutively active anti-sense ribosomal RNA (*rrn*) promoters into which the sequence to be transcribed into dsRNA is inserted. The sequences inserted into the plasmids for this study were derived from complementary DNA (cDNA) prepared using RNA extracted from tomato hornworm, *Manduca cinquemaculata*, a closely related species to *M. sexta*. The two dsRNA pTom vectors created in this study for plant transformation and dsRNA template synthesis were denoted dsvATP-L and dsvATP-S and contained inserted sequences of different length (2222 bp and 259 bp, respectively) targeting V-ATPase subunit A of *M. sexta*. The region covered by the shorter dsRNA sequence is contained within the longer dsRNA sequence (Fig 2.2). A dsRNA pTom vector denoted dsLacZ containing a 237 bp Beta-galactosidase (*lacZ*) dsRNA derived from *E. coli* was also used for attempted plant transformation. A dsRNA pTom vector denoted dsGFP containing a 223 bp green fluorescent protein (*gfp*) dsRNA derived from *Aequorea victoria* was used as a template for *in* vitro dsRNA synthesis.

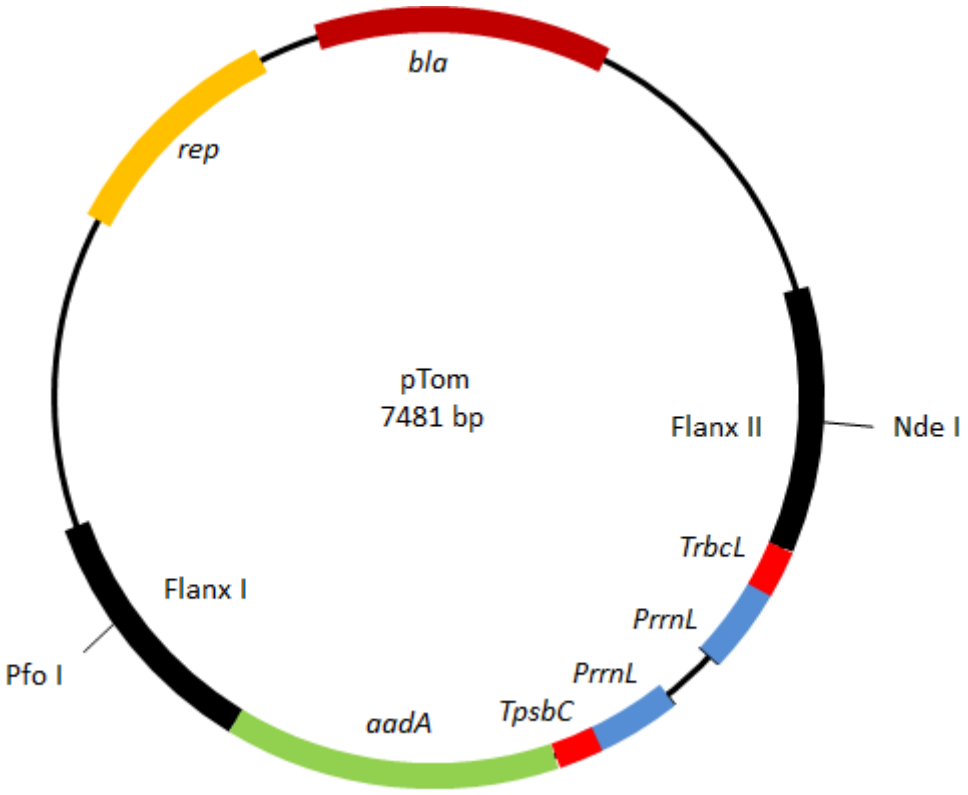

Figure 2.1. Map of empty pTom vector for tobacco chloroplast genome transformation. Indicated on the map are: *rep*, replication initiation protein; *bla*, ampicillin resistance gene; *aadA*, spectinomycin resistance gene; *TpsbC*/*TrbcL*, transcription terminator sequences; *PrrnL*, transcription initiation sequences; Flanx I/Flanx II, homologous recombination sequences; NdeI/PfoI, restriction enzyme digestion sites for Southern blot.

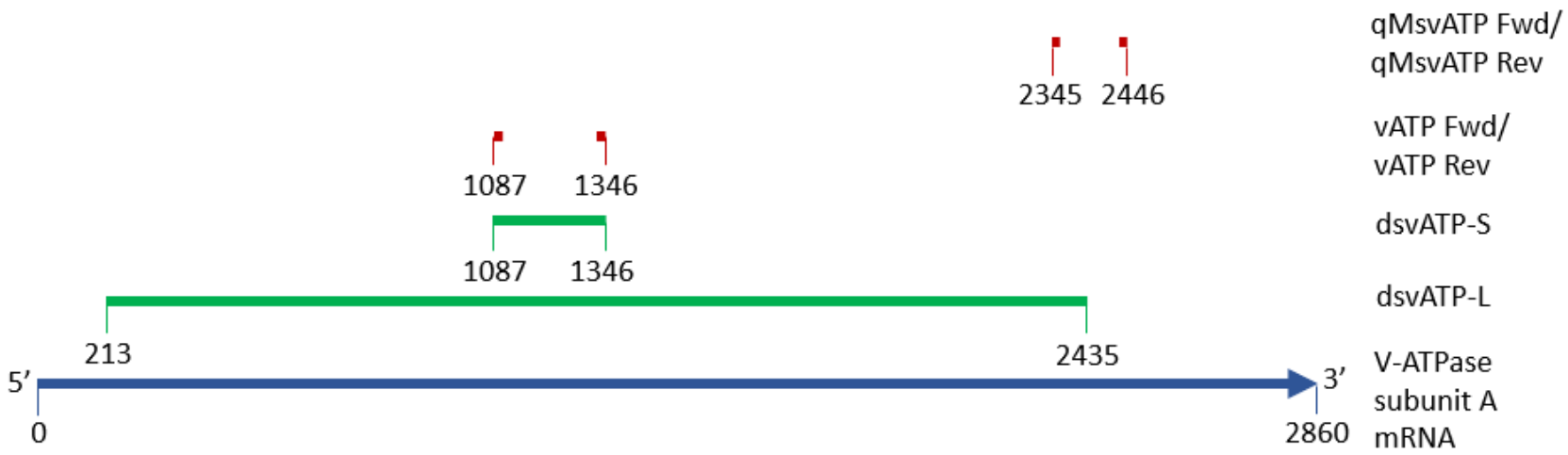

Figure 2.2. Coverage of double-stranded RNA regions compared to mRNA of V-ATPase subunit A. Values indicate the number of nucleotides from 5' end of mRNA. Primers used for detection of positive tobacco transformants and for quantitative RT-PCR of insect mRNA (Table 2.1) are also shown.

2.1.3. Plasmid Growth and Purification

Escherichia coli colonies containing the plasmid of interest were scraped and inoculated into 150 mL of LB medium containing 150 µL of 100 mg/mL ampicillin. Cultures were incubated overnight at 37ºC while shaking at 250 RPM. Plasmid DNA was extracted using the Quantum Prep Plasmid Midiprep Kit with 40 mL of culture per column. Purified plasmid DNA was concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (Millipore) and quantified with a NanoDrop Spectrophotometer (Thermo Scientific), then diluted with diH_2O to a final concentration of 1 µg/µL. Intact plasmid was confirmed through gel electrophoresis using a 1% (w/v) agarose gel.

2.1.4. Biolistic Particle Delivery

Biolistic particle delivery for chloroplast transformation was performed as follows. Methods were reported by Kolotilin *et al.* (2013) as derived from Svab *et al.* (1990a), Svab *et al.* (1990b), and Verma *et al.* (2008), with additional modifications suggested by Dr. Igor Kolotilin (personal communication).

Preparation of Gold Particles

Gold particles were vortexed with 1 mL of 100% ethanol for 2 min then centrifuged at 10,000 × g for 3 min. Supernatant was discarded and gold particles were re-suspended in 1 mL of 70% vol/vol ethanol. Particles were incubated at room temperature for 15 min while being mixed intermittently by shaking. Gold particles were again pelleted by centrifugation at 3,000 × g for 2 min. Supernatant was discarded and the resulting pellet was washed by re-suspending in 1 mL sterile diH_2O and incubated at room temperature for 1 min, followed by centrifugation at 5,000 × g for 2 min. This final wash step was repeated four times. Supernatant was discarded and gold particles were re-suspended and stored in 1 mL of sterile 50% v/v glycerol at -20ºC until needed.

Coating Gold Particles

The following protocol coated enough gold particles for five rounds of particle bombardment. Gold particles were re-suspended in 50% vol/vol glycerol by vortexing and 50 µL was aliquoted to a 1.5 mL Axygen microcentrifuge tube. While continuously vortexing, 5 µL of 1 µg/µL cDNA, 50 µL of 2.5 M $CaCl_2$, and 20 µL of 0.1 M spermidine were added. Vortexing was then continued for 20 min. Gold particles were centrifuged for 7,000 × g for 1 min. Supernatant was discarded, and the pellet was washed and re-suspended in 200 µL of 70% v/v ethanol, followed by centrifugation at 7,000 × g for 1 min. Supernatant was again discarded, and the pellet was washed and re-suspended in 200 µL of 100% ethanol, followed by centrifugation at 7,000 × g for 1 min. Supernatant was discarded, and the pellet was re-suspended in 70 µL of 100% ethanol. These particles were used immediately for bombardment.

Particle Bombardment

Five- to seven-week old sterile tobacco leaves were cut at their stems and placed on petri dishes containing RMOP (0.44% w/v MS Basal Salts with Vitamins, 3% w/v sucrose, 0.7% agar, 1 mg/L 6-benzylaminopurine, 0.1 mg/L 1-naphthaleneacetic acid, 1 mg/L thiamine hydrochloride, 0.1 g/L myo-Inositol) medium and Whatman 70-mm circle filter disks with the adaxial side of the leaf facing the media. Then, 13 µL of DNA-coated gold particles were loaded onto sterile macrocarriers, spread with a pipette tip, and then allowed to dry. Macrocarriers and Petri dishes containing leaves were loaded into a PDS-1000/He Particle Delivery System (Bio-Rad) according to the manufacturer's protocol. Approximately 1,400 psi of pressure was used for each leaf bombardment. After bombardment, plates containing leaves were held in darkness for 48 hrs then cut into 1 cm^2 pieces and dispersed onto plates containing 50 mL of RMOP + 0.05% w/v spectinomycin with the abaxial side facing the media for selection of transformants.

Table 2.1. List of primers used for PCR of templates for synthesis of dsRNA, DIG-labelled probe synthesis, PCR for confirmation of transgene insertion expression, and qPCR of insect RNA.

Primer name	Sequence (5'-3') (square brackets indicate T7 promoter sequence)
Primers used for dsRNA synthesis	
vATP-L-T7 Fwd	[TAATACGACTCACTATAGGG]AATCATCCGTCTTGAGGGTGA
vATP-L-T7 Rev	[TAATACGACTCACTATAGGG]CCACTGTGACGACTGTGTT
vATP-S-T7 Fwd	[TAATACGACTCACTATAGGG]ATGATGGCTGACTCGACCT
vATP-S-T7 Rev	[TAATACGACTCACTATAGGG]ACACCTGCACGATACCCAG
GFP-T7 Fwd	[TAATACGACTCACTATAGGG]ACTTTTCACTGGAGTTGTCCCAA
GFP-T7 Rev	[TAATACGACTCACTATAGGG]AGTAGTGACAAGTGTTGGCTGA
Primers used for confirmation of insertion/expression	
vATP Fwd	ATGATGGCTGACTCGACCT
vATP Rev	ACACCTGCACGATACCCAG
LacZ Fwd	CGAGTTGCGTGACTACCTA
LacZ Rev	GCTTCTGCTTCAATCAGCGT
GFP Fwd	ACTTTTCACTGGAGTTGTCCCAA
GFP Rev	AGTAGTGACAAGTGTTGGCTGA
NtEF1 Fwd	TTGGTCGTGTGGAAACTGG
NtEF1 Rev	CCAACATTGTCACCAGGAAGTG
Primers used for DIG-labelled probe synthesis	
DPNt Fwd	TCAATCCCTTTGCCCCTCAT
DPNt Rev	TCAACTGCCCCTATCGGAA
Primers used for qPCR of insect RNA	
qMsvATP Fwd	CAAAGGGCGTGGCTTGTAAA
qMsvATP Rev	ACCAACCCAGGTCCAAACAT
qMsEF1α Fwd	GTGTCCGTCAAGGAATTGCG
qMsEF1α Rev	TCCAGGGTGGTTGAGTACGA

2.1.5. Detection and Confirmation of Transformation

Putative transformants were detected by the presence of small, green calli protruding from leaf tissue incubated on RMOP + 0.05% w/v spectinomycin plates. Any observed calli were moved to fresh plates to allow for growth. If growth resumed, calli were cut into small pieces and dispersed onto fresh plates after 4-6 weeks. Leaf shoots that grew from calli were cut and re-dispersed onto fresh plates to again produce calli. This process was repeated twice. DNA was extracted after initial leaf growth using the DNeasy Plant Mini Kit (QIAGEN). PCR (94°C for 3 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s; 72°C for 7 min) was performed using primer pairs with sequence specificity to the dsRNA-encoding region of the transplastomic cassette (Table 2.1) and products were separated by electrophoresis on a 1.5% w/v agarose gel to confirm insertion of the expression cassette.

2.1.6. Confirmation of Homoplastomy

DNA Extraction

DNA was extracted from plant leaf tissue using CTAB Genomic DNA Extraction protocol (Porebski, 1997) as follows with modifications suggested from Dr. Igor Kolotilin. Cetyl trimethylammonium bromide (CTAB) buffer was prepared (2% w/v CTAB, 1.4 M NaCl, 100 mM Tris-HCl pH 7.5, 20 mM EDTA, 2 mM DTT). One hundred mg of leaf tissue were snap frozen in liquid nitrogen and pulverized in a TissueLyser II (QIAGEN) for 1 min at 30 Hz, then frozen and shaken again with the same settings. One mL of CTAB buffer was added to pulverized tissue and then heated to 65°C for 10 min. One mL of chloroform was added and the mixture vortexed followed by centrifugation at 12,000 × g at 4°C for 10 min. The aqueous layer was moved to a clean tube and 0.8 mL of cold isopropanol was added and mixed, followed by incubation at -80°C for 10 min. Samples were centrifuged for 15 min at 12,000 × g at 4°C and supernatant was removed. 1 mL of cold 70% ethanol was added to the pellet and vortexed followed by centrifugation at 12,000 × g at 4°C for 15 min and removal of the supernatant. Five hundred µL of RNase-TE (10mM Tris-Cl, 1mM EDTA, 10 µg/mL

RNase A) was added and the pellet was resuspended by swirling. Tubes were incubated at 55°C for 20 min. Thirty µL of 5M NaCl was added to each sample and mixed. Two-hundred fifty µL of 100% ethanol was added and mixed by inverting followed by incubation at -80°C for 10 min. Samples were centrifuged at 14,000 × g at 4°C for 10 min and supernatant was removed. Thirty µL of TE buffer was added and pellets were hydrated overnight at 4°C.

Southern Blot

Southern blotting protocol was adapted from Southern (2006), using a digoxigenin (DIG) antibody chemiluminescent visualization technique. The probe for Southern blotting was synthesized using the PCR DIG Probe Synthesis Kit (Sigma-Aldrich) protocol using primers from Table 2.1 with a PCR cycle of (94°C for 3 min; 35 cycles of 95°C for 30 s, 61°C for 30s, 72°C for 40 s; 72°C for 7 min). The probe was 1181 bp long and binds the Flanx I/II regions (Fig. 2.1). The probe was designed to span both Flanx regions and thus needed to be cut via restriction enzyme digestion. The probe was digested in a 50 µL reaction containing 50 U *Eco*RV and 1 × NEB1 buffer overnight at 37°C and digestion was stopped by 20 min incubation at 95°C. DNA samples from each plant used for bioassays were pooled together for each line (dsvATP-L, dsGFP, wild type). Double digestions using restriction enzymes *Pfo*I and *Nde*I (Fig 2.1) were performed. Reactions contained either 7 µg of genomic DNA for each line or 1 µg of each plasmid (pTom + dsvATP-L, pTom + dsGFP) in 20 µL containing 1 × Tango buffer and 20 U of each enzyme and were incubated at 37°C overnight. Reactions were stopped by incubation at 65°C for 20 min and then placed on ice. Each digest was separated on a 0.8% w/v agarose gel alongside 5 µL of DIG-labelled ladder IV (Sigma-Aldrich).

The gel was depurinated through wash with 500 mL of 0.2M HCl for 30 min. Gel was washed with 500 mL denaturing solution (0.5M NaOH, 1.5M NaCl) for 30 min, twice, to improve membrane binding. The gel was next washed with 500 mL of neutralizing solution (1M Tris-Cl, 1.5M NaCl, pH=8) for 30 min, twice, followed by washing with 500 mL of 10 × saline sodium-citrate (SSC) buffer (1.5M NaCl, 0.15M sodium citrate) for 15 min. Finally, transfer to a nitrocellulose membrane was set up as described by

Southern (2006) and allowed to run overnight. The membrane was crosslinked at 1200V using a CL-1000 Crosslinker (UVP). Membrane hybridization steps were performed in a rotary incubator at 8 RPM. The membrane was washed with 30 mL pre-hybridization buffer (73% w/v DIG Easy-Hyb Granules (Sigma-Aldrich)) for 1 hr at 50°C. The probe (synthesized above) was denatured at 98°C for 10 min then held on ice for 20 min and added to 20 mL pre-hybridization buffer (0.1% v/v probe). The membrane was incubated in this probe solution at 50°C to tag the complementary regions. The membrane was washed twice in 100 mL of 2 × SSC with 0.1% w/v sodium dodecyl sulfate (SDS) at room temperature for 15 min, then washed three times in 0.5 × SSC with 0.1% w/v SDS at 68^{o}C for 30 min, then washed with maleic acid buffer (0.1M maleic acid, 0.15M NaCl, pH=7.5) with 0.3% v/v Tween20 for at 45^{o}C for 5 min. The membrane was incubated in 100 mL blocking buffer (1% w/v Blocking Reagent (Sigma-Aldrich) in maleic acid buffer) at 48°C for 30 min to prevent non-specific antibody binding. The membrane was incubated in 4 mL of DIG-antibody solution (7.14% v/v Anti-Digoxigenin-AP Fab (Sigma-Aldrich)) at room temperature for 1 hr. The antibody binds to the DIG-labelled probe. The membrane was washed twice with maleic acid buffer containing 0.3% v/v Tween20 at room temperature for 15 min. The membrane was then submerged in detection buffer (0.1M Tris-HCl, 0.1M NaCl, pH=9.5) for 10 min. The membrane was covered in 2 mL detection buffer with 0.2% v/v CSPD, ready to use (Sigma-Aldrich). CSPD induces a chemilumiscent signal from DIG antibody. The signal was visualized using a MicroChemi 4.2 Bio Imaging System with a 60 min exposure time.

2.1.7. Confirmation of dsRNA Expression

RNA Extraction

RNA was extracted using a protocol from Deepa *et al.* (2014), with minor modifications, as follows. RNA extraction buffer was prepared containing (100mM Tris-HCl, 50mM EDTA, 2% w/v SDS, 0.1% w/v β-mercaptoethanol). Fifty mg of plant tissue were snap frozen in liquid nitrogen and shaken in a TissueLyser II (QIAGEN) at 30 Hz for 1 min, then frozen again and shaken again with the same settings. 0.1 mg of polyvinylpyrrolidone (PVP) was then added to the powderized tissue. One mL of

extraction buffer was added and vortexed to mix, then an equal volume of acid:phenol chloroform was added and vortexed. The mixture was held at room temperature for 10 min then centrifuged at 15,000 × g for 10 min at 4ºC. The supernatant was transferred to a fresh tube and 0.3 volume of 3M sodium acetate and 0.7 volume of acid:phenol chloroform were added followed by a 10 min incubation on ice and centrifugation at 15,000 × g for 10 min at 4ºC. The supernatant was transferred to a fresh tube and 0.1 volume 3M sodium acetate and 1 mL of cold isopropanol were added, followed by centrifugation at 15,000 × g for 10 min at 4ºC. The supernatant was discarded, and then the pellet was washed with 500 µL of cold 75% ethanol and centrifuged at 7,500 × g for 10 min at 4ºC. This washing step was repeated once. The supernatant was discarded, and then the pellet was air-dried and re-suspended in 30 µL RNase-free H_2O. RNA samples were treated with DNase to remove contaminating genomic DNA using the TURBO DNA-free Kit (Ambion) and intact product was confirmed through electrophoresis on a 1.5% agarose gel. RNA was quantified using a NanoDrop Spectrophotometer (Thermo Scientific).

Reverse Transcription PCR

cDNA for reverse transcription PCR (RT-PCR) was synthesized using a SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit using an equal amount of RNA from each extraction. An initial denaturation step of 5 min at 95°C followed by cooling on ice was added to ensure that dsRNA was denatured. Denaturation was performed in the presence of 1 µl of 50 M random hexamers to ensure annealing upon cooling. Reverse transcription controls (RTCs) containing all components other than enzyme were performed as well to check for genomic DNA carryover. PCR of each cDNA and RTC was performed using primers specific to *dsVATP-L*, *dsGFP*, or elongation factor 1 (*EF1*) (wild type control) (Table 2.1) followed by electrophoresis on a 1.5% w/v agarose gel to check for expression of each sequence in each plant.

2.1.8. Plant Growth and Seed Collection

After three rounds of selection and confirmation of homoplastomy, transplastomic tobacco calli were moved to magenta boxes containing MS media (0.44% w/v MS Basal Salts with Vitamins, 3% w/v sucrose, 0.7% w/v agar) and 0.05% w/v spectinomycin. Plants formed roots in this media in absence of the growth hormones present in RMOP. When plants grew to approximately 9 cm tall they were moved to pots containing soil for further growth. Plants on soil were fertilized once every two days with 0.05% w/v high nitrate fertilizer (20% nitrogen, 8% phosphoric acid, 20% potash). Plants were grown either in growth cabinets with ambient humidity and 16L:8D light cycles or in greenhouses. Plants were grown until buds began to develop and then the flowers were bagged to ensure self-pollination. Seeds were collected and sterilized by a 5 min wash with 70% ethanol, then dispersed onto plates containing MS medium with or without 0.05% w/v spectinomycin to observe whether T1 generation plants inherited the expression cassette. After seeds were collected, flowers were removed by cutting the top of the plant stem. This induced growth of basal shoots to produce leaf tissue for bioassays. After six weeks of growth, T1 seedlings were moved onto soil to observe growth in comparison to wild type plants.

2.2. *In vitro* Synthesis of dsRNA

PCR primers were designed to include T7 RNA polymerase promoter consensus sequences at the 5’ ends (Table 2.1). DNA templates for *in vitro* RNA synthesis were prepared using PCR with 100 ng of pTom plasmid containing the dsRNA-encoding sequence of interest as the template and a cycle profile of (94°C for 3 min; 35 cycles of 94°C for 30 s, 60°C for 30 s or 1.5 min (for dsGFP/dsvATP-S and dsvATP-L respectively), and 72°C for 30 s; 72°C for 7 min). The PCR products were separated via electrophoresis on a 1.5% w/v agarose gel and the expected size band was extracted and purified using QIAquick Gel Extraction Kit (QIAGEN). The extracted template was quantified using a NanoDrop spectrophotometer (Thermo Scientific). T7 transcription was performed using different kits for dsGFP/dsvATP-S and dsvATP-L dsRNA products. dsGFP/dsvATP-S synthesis was performed using the RiboMAX Express RNAi System

(Promega) with an incubation step of 3 hrs and approximately 1 µg of template per 10 µL of reaction mixture. dsvATP-L synthesis was performed using the MEGAscript T7 Transcription Kit (Ambion) with an incubation step of 3 hrs and approximately 1 µg of template per 20 µL reaction. dsvATP-L was further purified using protocol from the RNeasy Mini Kit (QIAGEN) to ensure no carry-over of enzymatic components. All dsRNA products were concentrated using an Amicon Ultra-15 Centrifugal Filter Unit 30K (Millipore) and quantified using a NanoDrop spectrophotometer (Thermo Scientific).

2.3. Insect Bioassays

2.3.1. Insect Rearing

Manduca sexta eggs were obtained from Reptile Feeders (Norwood, Ontario). For artificial diet bioassays, larvae were hatched in 5 oz plastic cups (Solo) containing approximately 10 g of artificial nicotine-free cornmeal-based diet per approximately 30 eggs. For *in planta* bioassays, larvae were hatched in cups containing 1% w/v agar to keep material moist, under a Whatman filter paper disc, and a wild type tobacco leaf disc having a diameter of about 10 cm, per approximately 30 eggs. Insects were reared at 27ºC, 70% relative humidity and an 18D:6L photoperiod.

2.3.2. Diet with dsRNA

dsRNA was prepared through *in vitro* synthesis as described above. Artificial, nicotine-free cornmeal-based diet was prepared. Insect diets were prepared by mixing dsvATP-L, dsvATP-S, and dsGFP dsRNA into cooled diet until a final concentration of 100 ng/mg of diet. Diets containing an equivalent volume of water were prepared as well. Twenty-five mg of diet were placed in individual wells of a 48-well plate and one <24 hr-old insect was added per well. Diet was replaced each day regardless of consumption and survival of insects after 1 week was recorded.

2.3.3. Droplet Feeding

Double-stranded RNA was prepared through *in vitro* synthesis as described above and diluted to a concentration of 2.5 µg/µL. *Manduca sexta* larvae were hatched and raised to second instar on artificial, nicotine-free cornmeal-based diet and then removed from food for six hrs. Starved insects were placed in front of 1 µL of dsRNA or water and observed to ensure the entire droplet was consumed. Any insects that did not consume the entire droplet were discarded. Immediately following consumption of the droplet, insects were moved to artificial diet and allowed to feed *ad libitum* for seven days. Mortality was recorded every 24 hrs post-droplet feeding over this period and modeled as Kaplan-Meier survival curves. Survival for treatments was compared using a log-rank test between each curve. To test for gene silencing, a separate group of insects was droplet-fed dsRNA for RNA extraction. A time interval of 3 days post-droplet feeding was selected for extraction due to the observation that the majority of deaths happened between days three and four.

RNA Extraction

RNA was extracted from insects using the RNeasy Mini Kit (QIAGEN) protocol for animal tissue. Whole insects were placed in 1.2 mL of Buffer RLT with 12 µL β-mercaptoethanol and homogenized in a high torque tissue grinder. Six hundred µL of ground tissue was passed through a QIAshredder spin column at 21,000 × g for 2 min. Extraction was performed as written in the manufacturer's protocol. RNA samples were treated with DNase using the TURBO DNA-free Kit (Ambion) to ensure no genomic DNA carryover, and intact product was confirmed by electrophoresis on a 1.5% agarose gel. RNA was quantified using a NanoDrop Spectrophotometer (Thermo Scientific).

Quantitative Reverse Transcription PCR

cDNA was synthesized using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR Kit. Quantitative reverse transcription PCR (qRT-PCR) was performed using SensiFAST SYBR No-ROX Mix Kit (Bioline) and a CFX96 Real-Time Detection System (Bio-Rad). Primers for RT-qPCR (Table 2.1) were designed to amplify approximately 100 bp regions of the *Manduca* V-ATPase subunit A gene. The amplified

regions were designed to be outside the coverage of the dsRNA product to avoid false positives attributed to dsRNA carryover during extractions. Primer pairs targeting elongation factor 1-alpha (*EF1α*) were prepared as well to serve as a reference gene. Primers were designed using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer amplification efficiencies were calculated using 2× diluted cDNA serially diluted 10× until a final concentration of 10^{-5}×. Dilution series PCR was performed with 10 µl samples in duplicate with each containing 2.5 µL of template and a two-step cycling profile (95°C for 2 min, 40 cycles of 95°C for 5 s and 60°C for 30 s for 40 cycles with fluorescence measured after each cycle) followed by melt-curve analysis (65-95°C for 5 s, increasing in 0.5^{o}C increments). No template controls (NTCs) were included for each reaction. Primers with efficiencies calculated between 95% and 105% by CFX Manager Software (Bio-Rad) were used for qRT-PCR. qRT-PCR for comparison of V-ATPase subunit A expression between insect samples fed different treatments was performed using 50× diluted cDNA and 4 µL per 10 µL well with each sample performed in triplicate. Statistical significance was calculated using one-way ANOVA and Dunnett's Test ($p<0.05$) for ΔΔCt values normalized to EF1α expression.

2.3.4. *In planta* Bioassay

Feeding chambers were prepared by adding 2 mL of 1% (w/v) agar covered by Whatman paper into 1.5 oz plastic cups (Solo). Leaf discs with a diameter of 3 cm were cut and placed on top of the Whatman paper. One insect was added per cup and 30 cups total were prepared for each transplastomic tobacco line tested. Food was replenished daily to allow insects to feed *ad libitum*. Mortality was measured every 24 hrs for seven days. This experiment was performed a second time with 20 insects for each treatment, for a total sample size of 50 insects per plant line. Survival percentages for each treatment were modeled using a Kaplan-Meier survival curve and compared using a log-rank test between each curve. After seven days of feeding, whole insect RNA was extracted using the methods described above. qRT-PCR was performed as described above to determine any changes in V-ATPase subunit A gene expression attributable to feeding on the transplastomic leaves.

Chapter 3: Results

3.1. Transplastomic Plant Development

3.1.1. Detection of transformants following biolistic particle delivery with a long V-ATPase dsRNA cassette

To determine whether the dsRNA expression cassette was present in genomic DNA from of a putative transformant, DNA was extracted and PCR was performed using primers specific to a 222 bp region the dsRNA-encoding sequence (Table 2.1) for the callus as well as wild type tissue. A sample of the pTom transformation plasmid containing the dsvATP-L cassette was used as a positive control. The transgenic sequence was detected in transformed, spectinomycin resistant tissue but not in wild type tissue (Fig. 3.1). The same test was performed for calli derived from dsvATP-S and dsLacZ bombardment using sequence specific primers (Table 2.1), but no product was detected in either case, indicating that any resistant tissue was a false positive (data not shown).

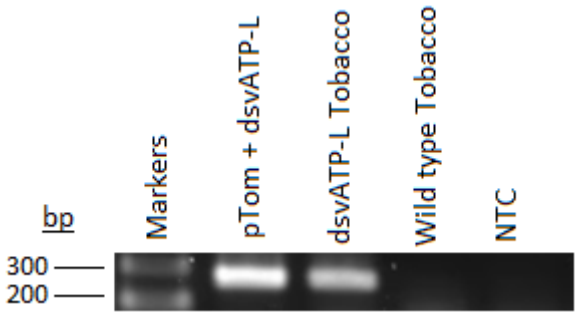

Figure 3.1. Confirmation of transgene insertion in chloroplast genome of *N. tabacum* following detection of an antibiotic-resistant callus. Whole plant DNA from a putative transformant and wild type tissue were extracted, and PCR was performed using *vATP* primers (Table 2.1) specific to the dsvATP-L sequence to detect insertion of the expression cassette.

3.1.2. Multiple rounds of selection induce homoplastomy of a dsvATP-L transformant

Following three rounds of selection and root induction on spectinomycin containing media, plants were moved to soil for further growth. Genomic DNA was extracted from all dsvATP-L plants used in insect bioassays as well as dsGFP plants obtained from Emine Kaplanoglu, and a Southern blot was performed. Digested pTom containing each dsRNA-encoding sequence were included as positive controls and wild-type tobacco DNA was included as a negative control. Each observed product for plant extractions was approximately the expected size (Fig. 3.2). The wild type tobacco product of around 2.1 kb represents the homologous recombination site without the insertion of an expression cassette. The approximately 2000 bp difference between the homoplastomic lines, dsvATP-L and dsGFP, are due to the difference in the size of the dsRNA-encoding regions.

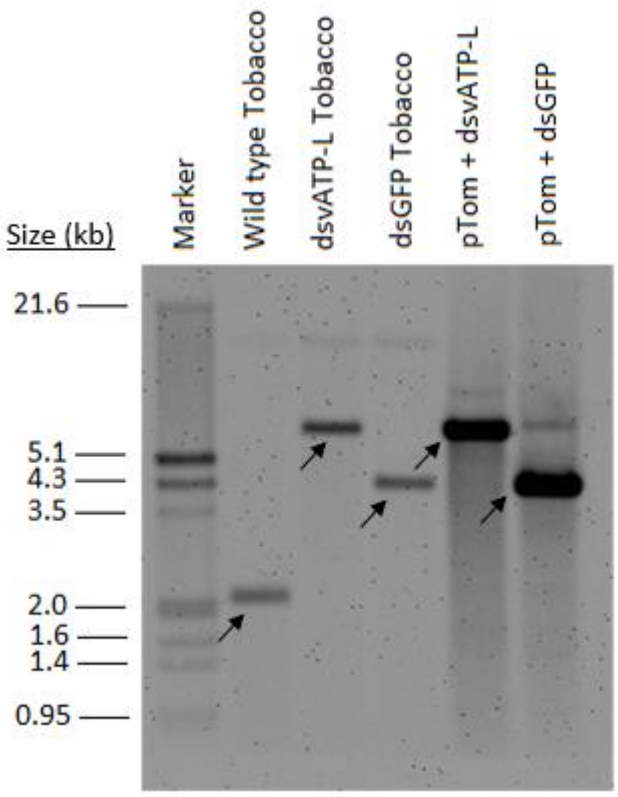

Figure 3.2. Southern blot for confirming homoplastomy of the transformed insertion region in each transplastomic tobacco line. Genomic DNA was extracted from each plant used for bioassays and pooled together according to their respective line. DNA and plasmids underwent digestion with restriction enzymes targeting the homologous recombination sites and were separated on an agarose gel followed by Southern blot protocol and hybridization with a probe representing the recombination region. Arrows indicate the expected bands. Additional bands in genomic DNA lanes are likely due to non-specific probe binding. Additional bands in pTom lanes are likely due to undigested plasmid.

3.1.3. dsRNA is expressed within transformed plants

RNA was extracted for each individual plant used in bioassays and reverse transcribed into cDNA, followed by PCR using primers specific to the dsRNA products in question. Integrity of the RNA extracted was confirmed using *EF1* expression as a positive control, and expression of the correct products for each respective transplastomic line was confirmed using dsRNA-specific primers (Fig. 3.3). RTCs were performed to ensure that DNA contamination did not carry over after RNA extraction, and no PCR products were obtained from these samples, indicating that the detected products are a result of mRNA expression (Fig. 3.3).

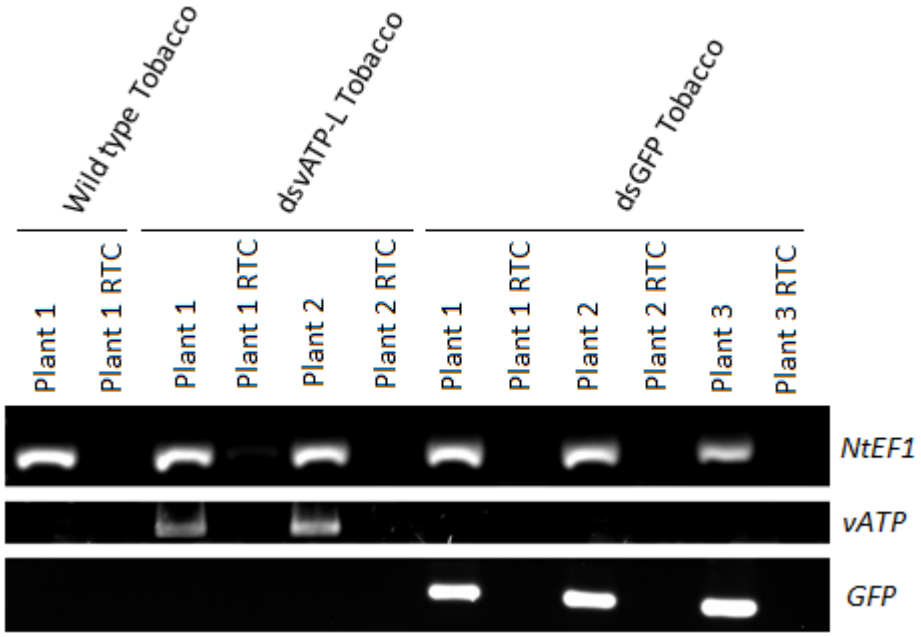

Figure 3.3. RT-PCR of RNA extracted from each plant used in bioassays. RNA was extracted from plant tissue and reverse transcribed into cDNA followed by PCR using primers targeting *EF1* (control), *dsvATP-L*, and ds*GFP* sequences (Table 2.1). Each reverse transcription was paired with an RTC to ensure that plastid DNA contamination was not being carried over into PCR.

3.1.4. Transgene is stable in subsequent generations

Seeds were obtained from transplastomic plants, sterilized and then placed onto MS medium containing spectinomycin. Seeds obtained from transplastomic plants were able to germinate and grow on antibiotic containing medium, indicating that the expression cassette had been passed on to the T1 generation, while wild type seedlings appeared bleached and did not grow beyond development of cotyledons (Fig. 3.4). Size disparity between seeds grown with and without spectinomycin is due to an initial lag phase during germination in the presence of antibiotic.

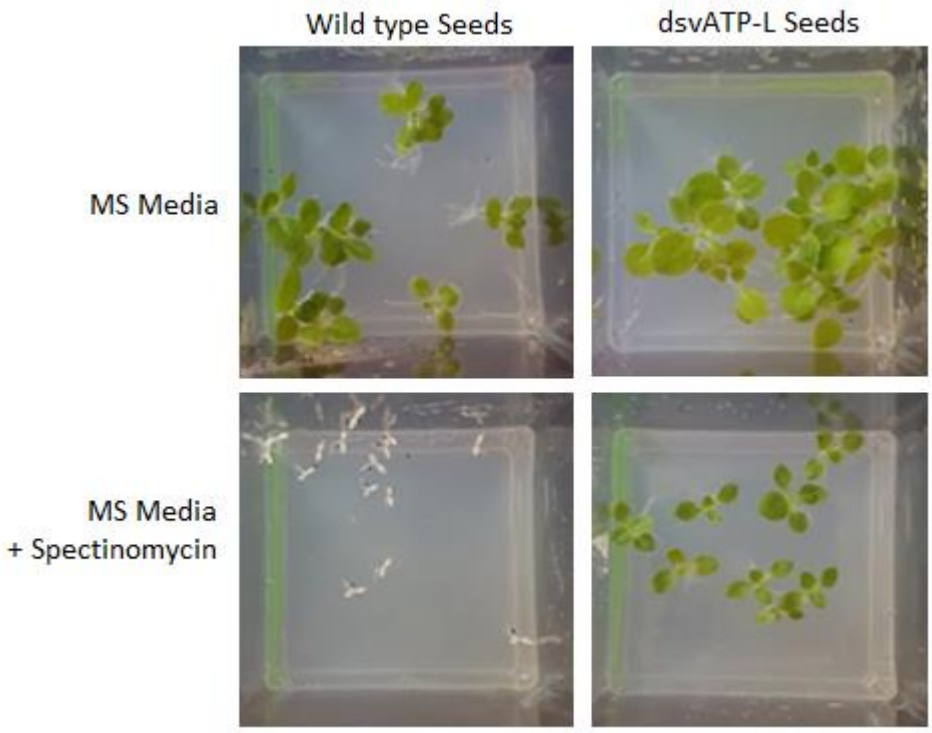

Figure 3.4. Transplastomic and wild type seed growth on media with and without selection. T1 seeds were obtained from flowering tobacco plants and sterilized, followed by dispersal onto growth media with or without spectinomycin.

3.1.5. Expression cassette insertion does not affect plant growth

T1 transplastomic tobacco plants were moved to soil after six weeks of growth in magenta boxes containing MS medium to observe growth relative to wild type plants. Beyond the initial lag phase on antibiotic medium, transplastomic plants grew at the same rate as wild type and appeared physically identical after ten weeks of growth, which suggests that expression cassette insertion and expression of the dsRNA does not affect the normal growth of the plant.

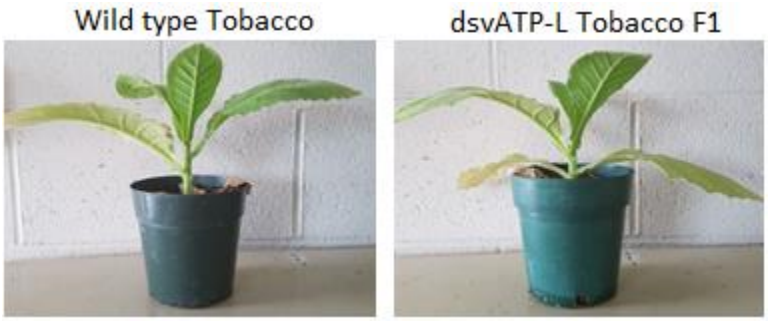

Figure 3.5. Growth of T1 tobacco plants on soil. T1 plants grown from dsvATP-L seeds were moved from magenta boxes containing spectinomycin to soil for further growth. Images were taken ten weeks post-germination.

3.2. *In vitro*-Synthesized dsRNA Bioassays

3.2.1. Droplet feeding affects survival of *M. sexta* larvae

Feeding *M. sexta* dsRNA mixed with artificial diet did not result in any effect on feeding insects (data not shown), and so a droplet feeding approach was used instead. Second instar *M. sexta* larvae were removed from artificial diet and starved for 6 hrs. Randomly chosen insects were then presented with a 1 µl droplet of either water alone or water containing 2.5 µg of dsGFP, dsvATP-S, or dsvATP-L, the sequence of the last two having been derived from *M. cinquemaculata*. Poreddy *et al.* (2017) found that using dsRNA sequences derived from *M. sexta* was sufficient for knockdown of gene expression of *M. cinquemaculata*, and so the inverse was assumed to be sufficient as well. After consuming the entire droplet, the insects were placed in individual wells where they were

fed artificial diet *ad libitum*. Figure 3.6 shows that insects fed with dsvATP-L had a higher mortality rate than insects fed water ($p < .001$) or dsGFP ($p = .019$), indicating that this dsRNA product affects survival of *M. sexta* larvae. dsvATP-S caused significant mortality in comparison to insects fed water ($p < .001$) but not insects fed non-target dsGFP ($p > .05$), suggesting that this product is not highly insecticidal. Examining the trends in Figure 3.6 suggests that the number of insect deaths peaked around day 2 for dsvATP-L and around days 4 and 5 for dsvATP-S, and that survival for all groups evens out after day 5. This suggests that the response of *M. sexta* larvae to droplet dsRNA ceases after about 5 days, or that all susceptible insects were killed prior to this point.

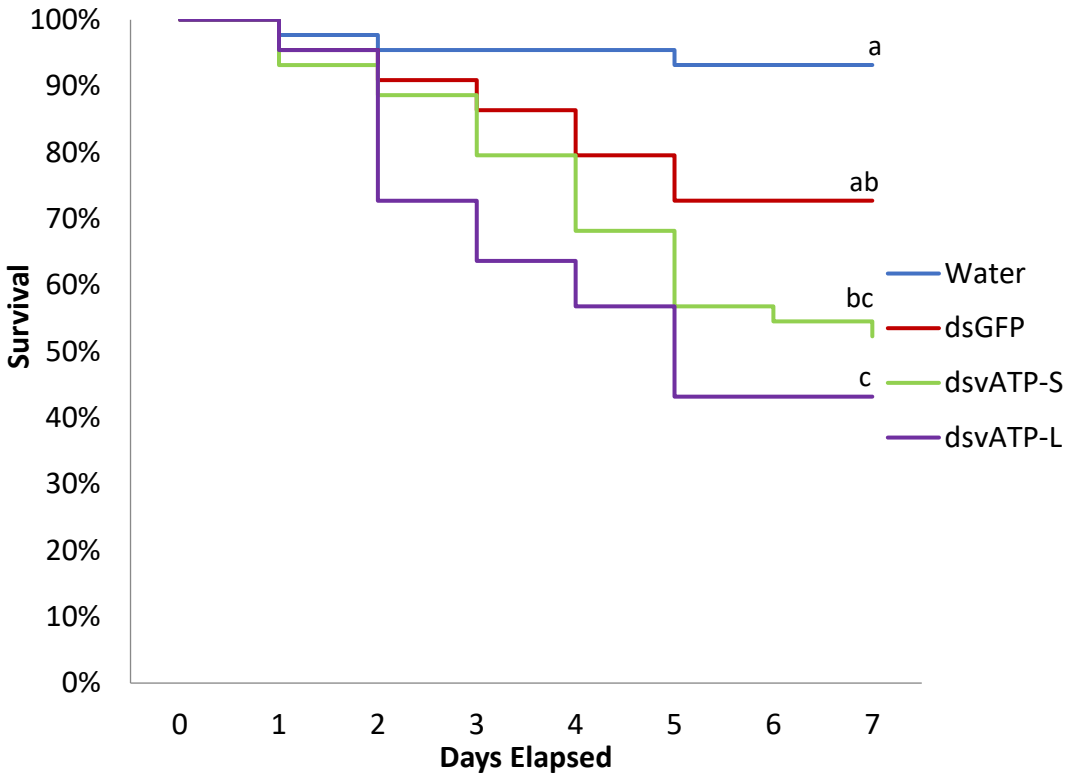

Figure 3.6. Kaplan-Meier survival curve indicating percent survival of insects on artificial diet each 24 hours post-dsRNA droplet feeding. Mortality was recorded every 24 hrs over a seven-day period (n=44). Different letters indicate significant difference between survival as calculated by log-rank tests between each treatment ($p<0.05$).

3.2.2. Droplet feeding with dsvATP RNA knocks down expression of V-ATPase subunit A mRNA in *M. sexta*

Four starved second instar larvae were fed a 1 µL droplet of solution containing 2.5 µg of either dsvATP-L, dsvATP-S, or dsGFP, or a 1 µL droplet of water, and then placed in individual wells and fed artificial diet *ad libitum*. Three days post droplet feeding, RNA was extracted from whole larvae and RT-qPCR was performed to compare knockdown of V-ATPase subunit A mRNA expression between treatments (Fig. 3.7). A significant knockdown effect was observed in insects fed dsvATP-L compared to insects fed a droplet of water (p = .042), whereas insects fed dsvATP-S dsRNA did not exhibit a significant knock down effect (p > .05). Observed mean knock down of expression for dsGFP, dsvATP-S, and dsvATP-L compared to control were 30.8%, 59.9%, and 67.9% respectively. This result provides an explanation for the effect on survival attributed to feeding on dsvATP-L seen in Figure 3.6.

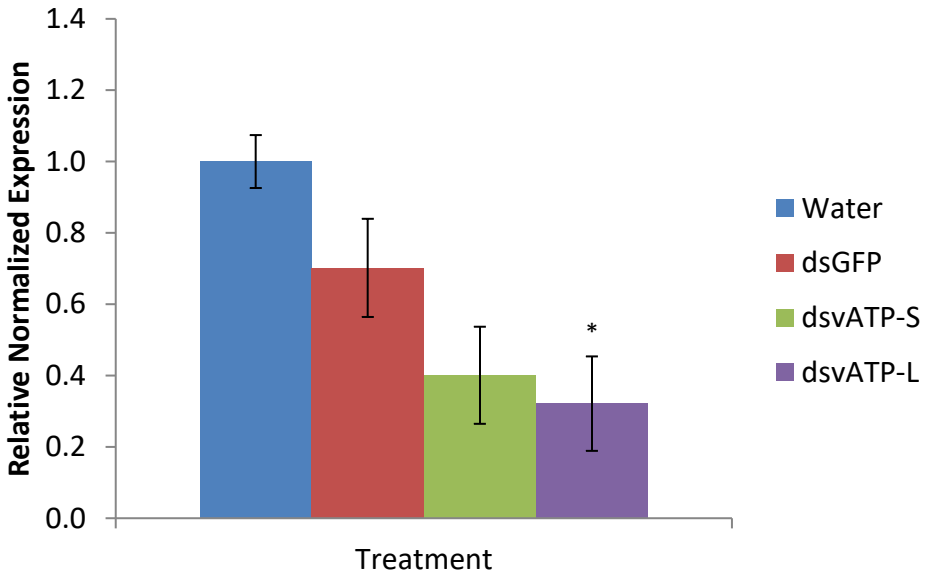

Figure 3.7. Expression of V-ATPase subunit A mRNA three days post-dsRNA droplet feeding. qRT-PCR was performed with V-ATPase subunit A-specific primers (Table 2.1) to measure relative expression in comparison to EF1α as a reference gene (± SEM, n=4). Normalized expression is set to 1.0 for control group (water). * = significant deviation from water treatment (Dunnett's test).

3.3. *In planta* Bioassays

3.3.1. Feeding on transplastomic plants is not lethal for *M. sexta* larvae

First instar *M. sexta* larvae were placed on leaf discs and permitted to feed *ad libitum* over a seven-day period. Survival was monitored, and mortality was recorded every 24 hrs for each tissue (Fig. 3.8). No effect on survival was observed as a consequence of feeding on transplastomic dsvATP-L plant tissue compared to wild type or dsGFP.

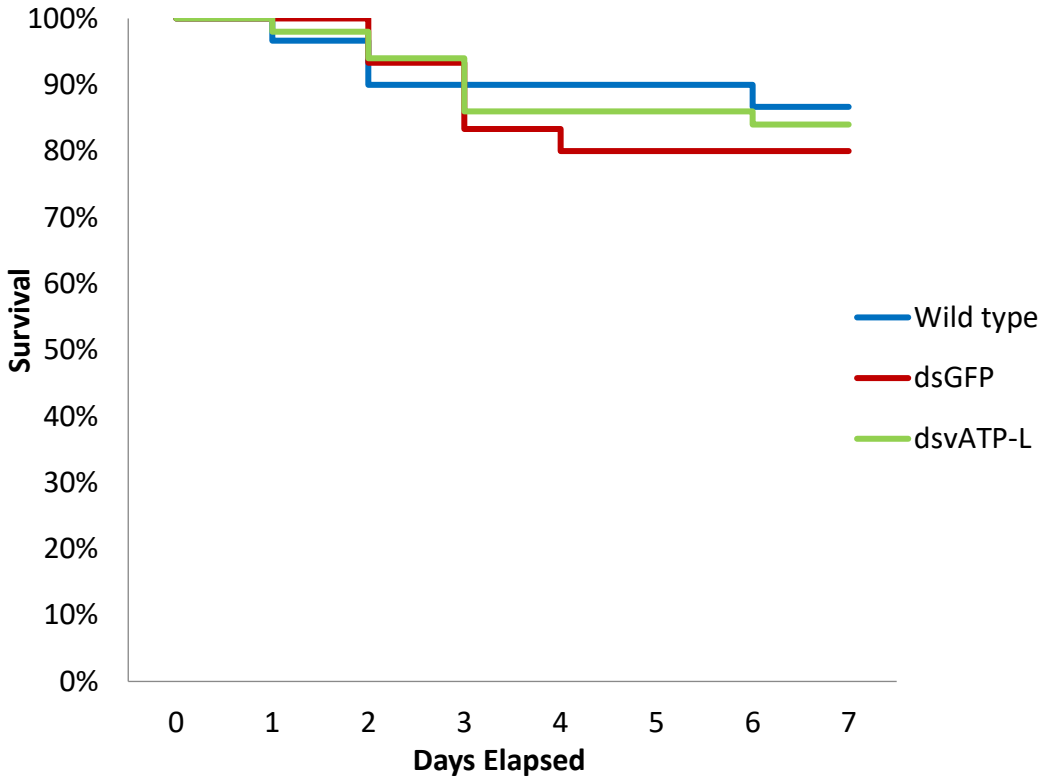

Figure 3.8. Kaplan-Meier survival curve indicating percent survival of insects on transplastomic and wild type tobacco. Mortality was recorded every 24 hours over a seven-day period (n=50).

3.3.2. Feeding on transplastomic plants does not affect expression of V-ATPase subunit A mRNA in *M. sexta* larvae

Whole insect RNA was extracted, and RT-qPCR was performed to measure any difference in expression of the V-ATPase subunit A gene attributed to feeding on transplastomic dsvATP-L plant tissue in comparison to wild type and dsGFP tissue. Although observed mean knockdown in insects feeding upon leaves from dsvATP-L plants was 28.8% compared to insects feeding upon wild type leaves, the variation between groups was not statistically significant ($p > .05$) (Fig. 3.9).

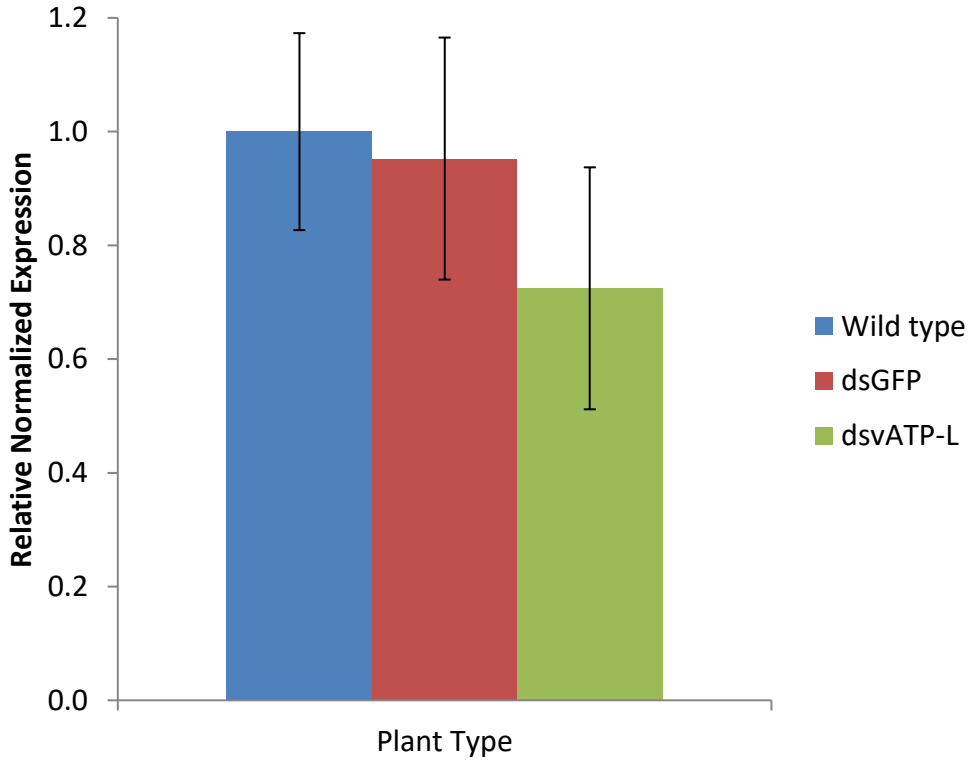

Figure 3.9. Expression of V-ATPase subunit A mRNA after seven days of feeding on transplastomic or wild type tobacco. qRT-PCR was performed with V-ATPase subunit A-specific primers (Table 2.1) to measure relative expression in comparison to EF1α as a reference gene (± SEM; n=8 for wild type and dsvATP-L, n=7 for dsGFP). Normalized expression is set to 1.0 for control group (wild type).

Chapter 4: Discussion

4.1. Gene-Specific Knockdown via *In vitro* dsRNA Feeding

Species within the order Lepidoptera are generally recalcitrant to applied dsRNA, especially in comparison with other orders of insects such as Diptera and Coleoptera. The first objective of this study was to determine whether dsRNA products with sequence complementarity to the V-ATPase subunit A mRNA of *M. sexta* were suitable for knocking down gene expression and inducing lethality in these insects through oral ingestion. An *in vitro* synthesized 2222 bp dsRNA product with this target successfully knocked down gene expression compared to a water control, and decreased survival of larvae in comparison to water and control dsRNA fed insects.

The initial approach for dsRNA application was through preparing artificial diet mixed with dsRNA so that insects would consume dsRNA consistently throughout the duration of the bioassay, as was successfully performed by Whyard *et al.* (2009) using *M. sexta* V-ATPase subunit E as a target. In this study, that approach had no observed effect on survival over a seven-day period. Deviations between studies in the effectiveness of RNAi targeting the same insect and even the same exact target are not uncommon. For example, Christiaens *et al.* (2014) attempted to replicate another finding by Whyard *et al.* (2009), which also reported successful gene knockdown and induction of lethality through feeding pea aphids *Acyrthosiphon pisum* with diet containing dsRNA targeting the *A. pisum* V-ATPase subunit E. Christiaens *et al.* (2014) observed the same survival in their dsRNA-fed insects as their control group using the lethal dose (LD_{50}) calculated by Whyard *et al.* (2009). Christiaens *et al.* (2014) also attempted to replicate an injection approach for dsRNA application in pea aphids reported by Mutti *et al.* (2006) and had a similar negative result. They suggested that RNAi response may have intraspecies variation and depend upon the strain of insects used in a given study. This hypothesis is supported by the findings reported here, and it is possible that not yet understood differences within species contribute to a lack of RNAi response. The recent discovery of a lepidopteran-specific nuclease that is upregulated in response to dsRNA exposure

(Guan *et al.*, 2018) indicates a mechanism for dsRNA degradation that could potentially differ between RNAi-resistant and -sensitive strains of a given species of insect, and so differences in expression of this protein between the two groups could be an important factor for consideration in a future study. Furthermore, intraspecies variation in dsRNA uptake by midgut cells could contribute to conflicting observations of RNAi response (Khajuria *et al*, 2018).

Droplet feeding of dsRNA successfully knocked down V-ATPase subunit A gene expression and induced lethality in *M. sexta* larvae. The success of this approach compared to the combined diet + dsRNA approach could depend on various factors. It is possible that a large quantity of dsRNA in a single application is crucial for a significant RNAi response to be observed in *M. sexta*. Degradation of dsRNA within the midgut and haemolymph of dsRNA insensitive insects (Wang *et al.*, 2016) remains an issue for researchers attempting to induce RNAi. The discovery of RNAses present in the midgut of lepidopteran species, specifically ones that are expressed and excreted exclusively by epithelial cells (Arimatsu *et al.*, 2007), is a likely explanation for the ineffective RNAi when consistently feeding dsRNA at relatively low concentrations. Applying a high concentration of dsRNA through a single droplet feeding step could be crucial for successful RNAi in these problematic insects. Because the nucleases which degrade dsRNA identified thus far seem to operate exogenously within the lumen of the midgut (Arimatsu *et al.*¸ 2007), it is possible that dsRNA remains intact once taken up by the cells of the epithelium. Therefore, a high enough concentration of dsRNA could saturate the available nucleases and allow for at least some intact product to be taken up and utilized by the RNAi pathway. Accumulation within endosomes following both SID-1-mediated (Shukla *et al.*, 2016) and endocytotic (Yoon *et al.*, 2017) dsRNA-uptake pathways presents another barrier that may require excessive amounts of dsRNA to overcome. Furthermore, the observation that midgut cells express extracellular receptors to dsRNA that initiate expression pathways (Liu *et al.*, 2013; Guan *et al.*, 2018) could provide another explanation as to why sustained feeding is less effective. It is possible that droplet feeding does not afford the insect an opportunity to upregulate its dsRNA-defense mechanisms, whereas exposure to dsRNA consistently may be less effective

immediately once the initial detection by these receptors upregulates production of these nucleases. Another possible explanation for the response variation could be that droplet-fed insects were starved prior to dsRNA application. The physiological composition of the midgut has been observed to change in response to starvation within insect species such as *D. melanogaster* (Li *et al.*, 2009) and *Periplaneta americana* (Mikani *et al.*, 2012), and to affect expression of specific genes influencing cell apoptosis in a lepidopteran, *Galleria mellonella* (Khoa and Takeda, 2012). It is possible that these nucleases or other insect dsRNA responses are downregulated as a consequence of starvation, which would increase the insect's susceptibility to ingested dsRNA. A future study could compare expression of dsRNA-responsive nucleases between starved and fed insects in response to dsRNA application. Furthermore, it is possible that droplet feeding may have induced RNAi outside of the midgut via the systemic pathway, and thus induced an enhanced insecticidal effect, which may not have occurred when low concentrations of dsRNA were fed. Whether systemic effects occur following consumption of dsRNA by lepidopterans is a controversial topic, but one study found that high concentration droplet feeding of *M. sexta* knocked down gene expression in chemosensory tissues of larvae, suggesting that a high enough dsRNA dose can induce a systemic response (Howlett *et al.*, 2012).

I found that a 2222 bp product was successful at knocking down expression of a target gene through oral ingestion by *M. sexta* and was at least as lethal as the use of a smaller 223 bp product. This appears to be the first reported instance of a >2000 bp dsRNA being used to successfully knock down gene expression in an insect. Previous studies have found that longer dsRNA products yield greater gene silencing due to a currently unknown mechanism that occurs subsequent to cellular uptake (Saleh *et al.*, 2006). Although lethality did not differ between application of the long and the short products, it is interesting to observe that the upper-limit on the size of dsRNA that can be taken up by midgut cells is at least 2222 bp. A possible benefit of using a large dsRNA product that was not investigated in this study is that more siRNA products are produced upon Dicer-mediated cleavage of the longer product compared to a shorter one, which means a greater number of mRNA targets can potentially be tagged. This could be beneficial in

field application as multiple insect species, or even an entire order, can be targeted through use of a longer product. This approach could also reduce the risk of insects evolving resistance through silent mutations in the targeted gene, as more regions will be susceptible, and therefore more mutations would be necessary to achieve resistance.

4.2. Development of Transplastomic Tobacco Expressing dsRNA

In the past, approaches to expressing insecticidal dsRNA through nuclear transformation of plants have been relatively ineffective compared to expectations, likely due to the processing of the insecticidal dsRNA into siRNA within plant cells prior to insect consumption. A possible solution is through the use of plastid genome transformation technology to insert transgenes expressing an insecticidal dsRNA product into the chloroplast genome of the plant for expression and intact storage within the plastid. In this study, I successfully transformed the chloroplast genome of tobacco with an expression cassette containing a 2222 bp dsRNA-encoding region and induced homoplastomy for the entire plant through repeated rounds of selection in tissue culture. Expression of the transgene was verified through RT-PCR and inheritability of the expression cassette into subsequent generations through seed germination on antibiotic was also confirmed.

An initial goal for this research was to express both a long and a short dsRNA product from the tobacco chloroplast genome with the goal of comparing any differences in expression efficiency and identifying any effects on RNAi effectiveness that could be associated with the size of the dsRNA product. Transformation efficiency using the methods described here were quite low, and attempts to insert the dsvATP-S cassette, as well as an 800 bp dsLacZ control cassette, into the tobacco chloroplast genome were unsuccessful. One surprising observation from this study is that the dsvATP-L transplastomic plant line was successfully generated while lines expressing shorter dsRNA products were not, as it was initially predicted that the larger cassette would be more likely to affect the structural integrity of the chloroplast genome, thus making it more difficult to stably transform. This result indicates that success with biolistic particle delivery remains inconsistent to some extent, as identical protocols using similar leaves

and plasmids resulted in unpredictable levels of success, and continuing enhancement of this technique is needed to improve its reliability and effectiveness.

A vital component of plant development in this research was to ensure homoplastomy of the transgene, which was confirmed through Southern blot analysis as well as by the observation that all F1 progeny grew in antibiotic media, indicating that random assortment of the transgene did not take place. Ensuring that all insertion regions in the plant tissue are transformed is an important aspect of developing transplastomic plants, especially when targeting a repeated region as was performed in this study, due to the ability of the chloroplast to repair DNA irregularities through homoplastomic recombination repair (Rowan *et al.*, 2010). Interestingly, some of the plant lines developed during this study lost the transgenic cassette at some point during growth, and reversion to wild type has been observed in other studies even after homoplastomy was assumed (Bohmert-Tatarev *et al.*, 2011). This indicates that it is important to perform DNA analysis or apply selection pressure intermittently during development and application of transplastomic plant lines.

Although expression of the dsRNA was confirmed through RT-PCR, the relative expression level of the product was not determined. Ideally, comparison between the long dsRNA line and short dsRNA line could have been performed, but typical methods for testing relative expression of a gene such as RT-qPCR were not suitable for measuring expression without the latter plants being available as a baseline. The amount of expression of a transgene within the chloroplast varies widely depending on the product, from less than 1% (Morgenfeld *et al.*, 2009) to as much as 70% (Oey *et al.*, 2009) total soluble protein in leaf tissue. The majority of transplastomic plant studies have focused on expression of protein products and thus the rate limiting factor could be either at the transcriptional or the translational level. If high level expression is dependent on transcription, then it is possible that the long dsRNA product was not highly expressed. Highly upregulated transcription of a >2000 bp product would require a significant allocation of resources, both in terms of nucleic acids and expression machinery, but Figure 3.5 shows that the phenotype of the transplastomic plants produced in this study was not affected, or at the very least growth was not stunted. Kolotilin *et al.* (2013)

theorized that insertion of a highly upregulated transgene disrupts chloroplast homeostasis resulting in stunted growth in some plant lines, an observation which was not seen here, possibly indicating that a high level of expression was not taking place.

4.3. Insect Feeding on Transplastomic Plants

Feeding transplastomic plants expressing dsRNA targeting vital genes has been shown to successfully knock down gene expression and affect the life cycle of two insects to date, the CPB (Zhang *et al.*, 2015) and the cotton bollworm (Jin *et al.*, 2015; Bally *et al.*, 2016). In my experiments, this approach was not successful at knocking down expression of V-ATPase subunit A in *M. sexta* feeding on transplastomic tobacco plants. As the results of this research suggest that the transformed plants express a dsRNA product to which *M. sexta* are susceptible in high doses, the issue of why these plants are ineffective in inducing RNAi likely lies either in the level of dsRNA expression within the plant or upon the mechanisms of insect uptake of dsRNA present in plant tissue.

As suggested previously, it is possible that the plant's ability to express dsRNA is limited by the size of the product. Prior to this study, the longest dsRNA product successfully expressed in chloroplast was 670 bp (Zhang *et al.*, 2015). Another possibility that could affect the amount of exposure for the insect is the relative level of stability of the dsRNA product within the chloroplast after expression. Bally *et al.* (2016) suggested that using hairpin loops rather than convergent promoters produces more stable dsRNA products *in planta*. Zhang *et al.* (2015) compared using hairpins and convergent promoters and found that the former strategy resulted in a greater total quantity but also a high number of truncated products. While expression of a stable long dsRNA product may have benefitted from a hairpin configuration, this approach would have introduced an additional challenge of transcribing an RNA product well over 4500 bp and may not have been technically feasible.

The other possible issues with chloroplast-encoded dsRNA and *M. sexta* are the highly alkaline lepidopteran midgut environment and other lepidopteran defenses toward dsRNA. Like the artificial diet + dsRNA experiment discussed previously, the *in planta*

feeding experiments did not have the added benefits of targeting starved insect with potentially altered midgut physiology. It is possible that the midgut cells of the insects detected dsRNA early in the feeding process and were able to upregulate nucleases to degrade the product within the gut, causing this approach to be ineffective. In the previous studies by Zhang *et al.* (2015), Jin *et al.* (2015), and Bally *et al.*, (2016), the targeted insects were CPB and cotton bollworm, both insects that have been shown to be susceptible to dsRNA through *in vitro* methods in previous studies (Baum *et al.*, 2007; Yang and Han, 2014). On the contrary, the midgut of *M. sexta* has been resistant in most approaches at sustained feeding of dsRNA, and this insect's midgut may simply be too difficult of a barrier to overcome using this strategy. Accumulation within endosomes due to insufficient dsRNA quantity remains a possible explanation for lack of response as well. It is possible that a sustained feeding experiment throughout the larval stage and observation into adulthood may have been necessary to determine the extent of the effect from feeding upon transplastomic plants. Jin *et al.* (2015) reported a decrease in pupation rate of about 27% when insects were fed on transplastomic plants expressing V-ATPase dsRNA but did not report an increase in larval mortality. In our experiments it was presumed that larval mortality would be observed within seven days due to success in past studies (Whyard *et al.* 2009).

No significant variation in V-ATPase subunit A expression was observed between groups of insects feeding on different transformed plant lines, however Figure 3.9 shows an approximately 30% reduction in mean expression between insects fed on dsvATP-L plants and wild type or dsGFP plants. Unfortunately, variation between samples resulted in a high margin of error, thus no evidence of gene silencing can be derived from this data. Variation could be attributed to differences in the amount of feeding between replicates or perhaps variation in expression within plant tissue. Within-species variation in RNAi response is also possible, as was suggested by Christiaens *et al.* (2014).

4.4. Future Directions

Mortality and gene expression knockdown of V-ATPase subunit A were not observed when *M. sexta* larvae fed upon transplastomic plants expressing a long dsRNA product targeting this gene. While the plants developed here are not necessarily suitable for application in pest management of *M. sexta,* there are some future directions that could be applied to the results from this paper to develop further knowledge. One experiment currently being performed is feeding of *M. sexta* larvae upon transplastomic tomato plants expressing a shorter 189 bp dsRNA targeting a hemipteran V-ATPase subunit A sequence which features sufficient sequence similarity to potentially knock down expression of this gene in *M. sexta*. If lethality or significant knockdown are observed through feeding on these plants, it can be assumed that the length of the 2222 bp dsRNA product is the limiting factor preventing a sufficient insecticidal result from taking place. A second experiment that could be performed is the addition of EDTA to transplastomic tobacco leaves for insect feeding. As midgut RNases appear to be metal dependant, past studies have used EDTA to inhibit their activity (Barra *et al.*, 2015). EDTA is a chelating agent and can sequester metal ions, thus lowering the insect's ability to resist foreign dsRNA. Future studies should also consider investigating within-species variation in RNAi response, as this trait could explain the differences in results observed by different researchers studying the same insect species. For example, Khajuria *et al.* (2018) identified a population of *Diabrotica virgifera*, a species that is generally sensitive to RNAi, which displayed resistance to all orally administered dsRNA. They determined that these insects lacked the ability to uptake dsRNA into their midgut cells, suggesting that mutations in transmembrane domains could confer RNAi-resistance in specific insect populations.

4.5. Conclusion

In this study, I determined that expression of a long dsRNA in tobacco chloroplast was not a successful method for inducing RNAi driven lethality in *M. sexta* larvae feeding upon the plant. These insects are generally recalcitrant towards RNAi through oral exposure due to the presence of nucleases excreted within their gut which break down

ingested nucleic acids, and it is likely that this barrier prevented the chloroplast-expression method from being particularly effective. Alternatively, the decision to use a long dsRNA may have prevented accumulation of large amounts of dsRNA in the chloroplasts. Although other research has suggested that chloroplast-encoded dsRNA is a promising method for developing insecticidal crops, future studies should be aware of the potential limitations of this strategy and ensure that product design, gene target, and insect target are carefully considered to avoid similar negative results.

References

Agriculture and Agri-Food Canada. (2013). We grow a lot more than you think. Retrieved from http://www.agr.gc.ca/eng/about-us/publications/we-grow-a-lot-more-than-you-may-think/?id=1251899760841 (Accessed 27 June 2018).

Allen, M.L. and Walker, W.B. (2012). Saliva of *Lygus lineolaris* digests double-stranded ribonucleic acids. *Journal of Insect Physiology,* 58(3): 391-396.

Alyokhin, A., Baker, M., Mota-Sanchez, D., Dively, G., and Grafius, E. (2008). Colorado potato beetle resistance to insecticides. *American Journal of Potato Research*, 85(6): 395-413.

Arenas, I., Bravo, A., Soberón, M., and Gómez, I. (2010). Role of alkaline phosphatase from *Manduca sexta* in the mechanism of action of *Bacillus thringiensis* Cry1Ab toxin. *Journal of Biological Chemistry*, 285: 12497-12503.

Arimatsu, Y., Kotani, E., Sugimura, Y., and Furusawa, T. (2007). Molecular characterization of a cDNA encoding extracellular dsRNase and its expression in the silkworm, *Bombyx mori*. *Insect Biochemistry and Molecular Biology*, 37(2): 176-183.

Aronstein, K., Pankiw, T., and Saldivar, E. (2006). SID-1 is implicated in systemic gene silencing in the honey bee. *Journal of Apicultural Research*, 45: 20-24.

Bally, J., McIntyre, G.J., Doran, R.L., Lee, K., Perez, A., and Jung, H. (2016). In-plant protection against *Helicoverpa armigera* by production of long hpRNA in chloroplasts. *Frontiers in Plant Science,* 7: 1453.

Barra, G.B., Rita, T.H.S., Vasques, J.D., Chianca, F.C., Nery, L.F.A., and Costa, S.S.S. (2015). EDTA-mediated inhibition of DNases protects circulating cell-free DNA from ex vivo degradation in blood samples. *Clinical Biochemistry*, 48(15): 976-981.

Baum, J.A., Bogaert, T., Clinton, W., Heck, G., Feldmann, P., and Ilgan, O. (2007). Control of coleopteran insect pests through RNA interference. *Nature Biotechnology*, 25(110: 1322-1327.

Bencko, V. and Foong, F. (2017). The history of arsenical pesticides and health risks related to the use of Agent Blue. *Annals of Agricultural and Environmental Medicine*, 24(2): 312-316.

Bernstein, E., Caudy, A.A., Hammond, S., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, 409: 363-365.

Bohmert-Tatarev, K., McAvoy. S., Daughtry, S., Peoples, O.P., and Snell, K.D. (2011). High levels of bioplastic are produced in fertile transplastomic tobacco plants engineered with a synthetic operon for the production of polyhydroxybutyrate. *Plant Physiology*, 155: 1690-1708.

Boyhan, D. and Daniell, H. (2011). Low-cost production of proinsulin in tobacco and lettuce chloroplasts for injectable or oral delivery of functional insulin and C-peptide. *Plant Biotechnology Journal*, 9(5): 585-598.

Brévault, T., Tabashnik, B.E., and Carrière, Y. (2015). A seed mixture increases dominance of resistance to Bt cotton in *Helicoverpa zea*. *Scientific Reports*, 5: 9807.

Buzzetti, K.A., Chorbadjian, R.A., Fuentas-Contreras, E., Gutierrez, M., Rios, J.C., and Nauen, R. (2016). Monitoring and mechanisms of organophosphate resistance in San Jose scale, *Diaspidiotus pernicosus* (Hemiptera: Diaspididase). *Journal of Applied Entomology*, 140: 307-316.

Cahill, M., Gorman, K., Day, S. and Denholm, I. (2009). Baseline determination and detection of resistance to imidacloprid in *Bemisia tabaci* (Homoptera: Aleyrodidae). *Bulletin of Entomological Research*, 86(4): 343-349.

Cancino-Rodezno, A., Alexander, C., Villaseñor, R., Pacheco, S., Porta, H., Pauchet, Y., Soberón, M., Gill, S.S., and Bravo, A. (2010). The mitogen-activated protein kinase p38 is involved in insect defense against Cry toxins from *Bacillus thuringiensis*. *Insect Biochemistry and Molecular Biology*, 40(1): 58-63.

Cappelle, K., de Oliveira, C.F., van Eynde, B., Christiaens, O., and Smagghe, G. (2016). The involvement of clathrin-mediated endocytosis and two Sid-1-like transmembrane proteins in double-stranded RNA uptake in the Colorado potato beetle midgut. *Insect Molecular Biology*, 25(3): 315-323.

Casanova, H., Ortiz, C., Peláez, C., Vallejo, A., Moreno, M.E., and Acevedo, M. (2002). Insecticide formulations based on nicotine oleate stabilized by sodium caseinate. *Journal of Agricultural and Food Chemistry*, 50: 6389-6394.

Chatterjee, S. and Riaz, H. (2013). Death by insecticide. *BMJ*, 346: f2029.

Christiaens, O., Swevers, L., and Smagghe, G. (2014). DsRNA degradation in the pea aphid (*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay. *Peptides*, 53: 307-314.

Clark, T.M. (1999). Evolution and adaptive significance of larval midgut alkalinisation in the insect superorder mecopterida. *Journal of Chemical Ecology*, 25(8): 1945-1960.

Contreras, H.L., Goyret, J., von Arx, M., Pierce, C.T., Bronstein, J.L., Raguso, R.A., and Davidowitz, G. (2013). The effect of ambient humidity on the foraging behavior of the hawkmoth *Manduca sexta*. *Journal of Comparative Physiology*, 199(11): 1053-1063.

Daniell, H., Vivekananda, J., Nielsen, B.L., Ye, G.N., Tewari, K.K., and Sanford, J.C. (1990). Transient foreign gene expression on chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. *Proceedings of the National Academy of Sciences of the United States of America*, 87: 88-92.

Deepa, K., Sheeja, T.E., Santhi, R., Sasikumar, B., Cyriac, A., Deepesh, P.V., and Prasath, D. (2014). A simple and efficient protocol for isolation of high quality functional RNA from different tissues of turmeric (*Curcuma longa* L.). *Physiology and Molecular Biology of Plants*, 20(2): 263-271.

del Campo, M.L., Miles, C.I., Schroeder, F.C., Mueller, C., Booker, R., and Renwick, J.A. (2001). Host recognition by the tobacco hornworm is mediated by a host plant compound. *Nature*, 411: 186-189.

Dietzl, G., Chen, D., Schnorrer, F., Su, K., Barnova, Y., and Fellner, M. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature*, 7150: 151.

Doench, J.G., Petersen, C.P., and Sharp, P.A. (2003). siRNAs can function as miRNAs. *Genes and Development*, 17(4): 438-442.

Dong, Y. and Friedrich, M. (2005). Nymphal RNAi: systemic RNAi mediated gene knockdown in juvenile grasshopper. *BMC* Biotechnology, 5: 25.

Dow, J.A. (1992). pH gradients in lepidopteran midgut. *Journal of Experimental Biology*, 172: 355-375.

Duan, J.J., Marvier, M., Huesing, J., Dively, G., and Huang, Z.Y. (2008). A meta-analysis of effects of Bt crops on honey bees (Hymenoptera: Apidae). *PLoS One*, 3(1): e1415.

Eleftherianos, I., Millichap, P.J., ffrench-Constant, R.H., and Reynolds, S.E. (2006). RNAi suppression of recognition protein mediated immune responses in the tobacco hornworm *Manduca sexta* causes increased susceptibility to the insect pathogen Photorhabdus. *Developmental & Comparative Immunology*, 30(12): 1099-1107.

Esson, K.M., and Leeder, S.R. (2004). The Millenium development goals and tobacco control: an opportunity for global partnership. *World Health Organization*. Retrieved from http://www.who.int/tobacco/publications/mdg_final_for_web.pdf (Accessed: 27 June 2018).

Fabrick J.A. (2010). Insights into the genetics and molecular mechanisms of pink bollworm resistance to cry toxins. *Southwestern Entomologist* 35(3): 431-435.

Faure, S., Noyer, J., Carreel, F., Horry, J., Bakry, F., and Lanaud, C. (1994). Maternal inheritance of chloroplast genome and paternal inheritance of mitochondrial genome in bananas (*Musa acuminate*). *Current Genetics*¸ 25(3): 265-269.

Feinberg, E.H. and Hunter, C.P. (2003). Transport of dsRNA into cells by the transmembrane protein SID-1. *Science*, 301(5639): 1545-1547.

Fire, A., Albertson, D., Harrison, S.W., and Meorman, D.G. (1991). Production of antisense RNA leads to effective and specific inhibition of gene expression in *C. elegans* muscle. *Development*, 113(2): 503-514.

Fire, A., Xu, S., Montgomery, M., Kostas, S.A. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391: 806-811.

Flores-Escobar, B., Rodríguez,-Magadan, H., Bravo, A., Soberón, M., and Gómez, I. (2013). Differential role of *Manduca sexta* aminopeptidase-N and alkaline phosphatase in the mode of action of Cry1Aa, Cry1Ab, and Cry1Ac toxins from *Bacillus thuringiensis*. *Applied and Environmental Microbiology*, 79(15): 4543-4550.

Food and Agriculture Organization of the United Nations. (2003). Projections of tobacco production, consumption and trade to the year 2010. Retrieved from http://www.fao.org/3/a-y4956e.pdf (Accessed: 27 June 2018).

Gao, H., Zhang, Y., Zhao, Y., Qiu, C., Liu, J., Yang, J., Hua, Y., Yang, S., Liu, J., Liu, Z., and Hou, Y. (2016). Complete chloroplast genome sequence of *Nicotiana tabacum* TN90 (Solanaceae). *Mitochondrial DNA Part B*, 1(1): 867-868.

Garbutt, J.S., Bellés, X., Richards, E.H., and Reynolds, S.E. (2013). Persistence of double-stranded RNA in insect hemolymph as a potential determiner of RNA interference success: evidence from *Manduca sexta* and *Blattella germanica*. *Journal of Insect Physiology*, 59(2): 171-178.

Gastony, G.J. and Yatskievych, G. (1992). Maternal inheritance of the chloroplast and mitochondrial genomes in cheilanthoid ferns. *American Journal of Botany*, 79: 716-722

Gelvin, S.B. (2003). Agrobacterium-mediated plant transformation: The biology behind the "gene-jockeying" tool. *Microbiology and Molecular Biology Reviews*, 67(1): 16-37.

Ghosh, S.K.B., Hunter, W.B., Park, A.L. and Gundersen-Rindal, D.E. (2018). Double-stranded RNA oral delivery methods to induce RNA interference in phloem and plant-sap-feeding hemipteran insects. *Journal of Visualized Experiments*, 135: e57390.

Gómez, I., Flores, B., Bravo, A., and Soberón, M. (2015). Bacillus thuringiensis Cry1AbMod toxin counters tolerance associated with low cadherin expression but not that associated with low alkaline phosphatase expression in *Manduca sexta*. *Peptides*, 68: 130-133.

Gorantala, J., Grover, S., Rahi, A., Chaudhary, P., Rajwanshi, R., Sarin, N.B., and Bhatnagar, R. (2014). Generation of protective immunte response against anthrax by oral immunization with protective antigen plant-based vaccine. *Journal of Biotechnology*, 176: 1-10.

Gringorten, J.L., Crawford, D.N., and Harvey, W.R. (1993). High pH in the ectoperitrophic space of the larval lepidopteran midgut. *Journal of Experimental Biology*, 183: 353-359.

Guan, R.B., Li, H.C., Fan, Y.J., Hu, S.R., Christiaens, O., Smagghe, G., and Miao, X.X. (2018). A nuclease epsecific to lepidopteran insects suppresses RNAi. *The Journal of Biological Chemistry*, 293(16): 6011-6021.

Hamblin, J.D. (2015). Pick your poison. *Science*, 347(6227): 1208.

Horsch, R.B., Fry, J.E., Hoffman, N.L., Eichholtz, D., Rogers, S.G., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. *Science*¸ 227(4691): 1229-1231.

Hossain, A.M. and Salehuddin, S.M. (2013). Analytical determination of nicotine in tobacco leaves by gas chromatography-mass spectrometry. *Arabian Journal of Chemistry*, 6(3): 275-278.

Hunter, C.P., Winston, W.M., Molodowitch, C., Feinberg, E.H., Shih, J., Sutherlin, M., Wright, A.J., and Fitzgerald, M.C. (2006). Systemic RNAi in *Caenorhabditis elegans*. *Cold Spring Harbor Symposia on Quantitative Biology*, 71: 95-100.

Hutvágner, G. and Zamore, P.D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. *American Association for the Advancement of Science*, 297: 2056-2060.

Huvenne, H. and Smagghe, G. (2010). Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. *Journal of Insect Physiologyi,* 56(3): 227-235.

Jin, S., Singh, N.D., Li, L., Zhang, X., and Daniell, H. (2015). Engineered chloroplast dsRNA silences cytochrome p450 monooxygenase, V-ATPase and chitin synthase genes in the insect gut and disrupts *Helicoverpa armigera* larval development and pupation. *Plant Biotechnology Journal*, 13(3): 435-446.

Khajuria, C., Ivashuta, S., Wiggins, E., Flagel, L., Moar, W., Pleau, M., Miller, K., Zhang, Y., Ramaseshadri, P., Jiang, C., Hodge, T., Jensen, P., Gowda, A., McNulty, B., Vazquez, C., Bolognesi, R., Haas, J., Head, G., and Clark, T. (2018). Development and characterization of the first dsRNA-resistant insect population from western corn rootworm, *Diabrotica virgifera virgifera* LeConte. *PLoS One.* 13(5): e0197059.

Khoa, D.B. and Takeda, M. (2012). Expression of autophagy 8 (Atg8) and its role in the midgut and other organs of the greater wax moth, *Galleria mellonella,* during metamorphic remodelling and under starvation. *Insect Molecular Biology*¸ 21(5): 473-487.

Killiny, N., Hajeri, S., Tiwari, S., Gowda, S., and Stelinski, L.L. (2014). Double-stranded RNA uptake through topical application, mediates silencing of five CYP4 genes and suppresses insecticide resistance in *Diaphorina citri*. *PLoS One*, 9(10): e110536.

Kingsolver, J.G. and Nagle, A. (2007). Evolutionary divergence in thermal sensitivity and diapause of field and laboratory populations of *Manduca sexta*. *Physiological and Biochemical Zoology*, 80(5): 473-479.

Kislev, M.E., Hartmann, A., and Bar-Yosef, O. (2006). Early domesticated fig in the Jordan Valley. *Science*, 312(5778): 1372-1374.

Kolotilin, I., Kaldis, A., Pereira, E.O., Laberge, S., and Menassa, R. (2013). Optimization of transplastomic production of hemicellulases in tobacco: effects of expression cassette configuration and tobacco cultivar used as production platform on recombinant protein yields. *Biotechnology for Biofuels*, 6: 65.

Kumar, P., Pandit, S.S., and Baldwin, I.T. (2012). Tobacco rattle virus vector: A rapid and transient means of silencing *Manduca sexta* genes by plant mediated RNA interference. *PLoS One*, 7(2): e31347.

Kumar, P., Pandit, S.S., Stepphun, A., and Baldwin, I.T. (2014). Natural history-driven, plant-mediated RNAi-based study reveals CYP6B46's role in a nicotine-mediated antipredator herbivore defense. *Proceedings of the National Academy of Sciences of the United States of America*, 111(4): 1245-1252.

Lakshmi, P.S., Verma, D., Yang, X., Lloyd, B., and Daniell, H. (2013). Low cost tuberculosis vaccine antigens in capsules: Expression in chloroplasts, bio-encapsulation, stability, and functional evaluation in vitro. *PLoS One*, 8(1): e54708.

Lavine M.D. and Beckage, N.E. (1996). Temporal pattern of parasitism-induced immunosuppression in *Manduca sexta* larvae parasitized by *Cotesia congregate*. *Journal of Insect Physiology*, 42(1): 41-51.

Leuschner, P.J.F., Ameres, S.L., Kueng, S., and Martinez, J. (2006). Cleavage of the siRNA passenger strand during RISC assembly in human cells. *EMBO Reports*, 7(3): 314-320.

Li, H.M., Sun, L., Mittapalli, O., Muir, W.M., Xie, J., Wu, J., Schemerhorn, B.J., Sun, W., Pittendrigh, B.R., and Murdock, L.L. (2009). Transcriptional signatures in response to wheat germ agglutinin and starvation in *Drosophila melanogaster* larval midgut. *Insect Molecular Biology*, 18(1): 21-23.

Li, Y., Kai, Z., Huang, J., and Tobe, S.S. (2017). Lepidopteran HMG-CoA reductase is a potential selective target for pest control. *PeerJ*, 5: e2881.

Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L., Hannon, G.J. (2004). Argonaute2 is the catalytic engine of mammalian RNAi. *Science*, 305(5689): 1437-1441.

Liu, J., Smagghe, G., and Swevers, L. (2013). Transcriptional response of BmToll9-1 and RNAi machinery genes to exogenous dsRNA in the midgut of *Bombyx mori*. *Journal of Insect Physiology*, 59: 646-654.

Madanala, R., Gupta, V., Pandey, A.K., Srivastava, S., Pandey, V., Singh, P.K., and Tuli, R. (2015). Tobacco chloroplasts as bioreactors for the production of recombinant superoxide dismutase in plants, an industrially useful enzyme. *Plant Molecular Biology Reporter*, 33(4): 1107-1115.

Mao, Y., Cai, W., Wang, J., Hong, G., Tao, X., and Wang, L. (2007). Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nature Biotechnology*, 25(11): 1307-1310.

Mattila, H.R., Siegfried, B.D., and Dively, G.P. (2001). Impact of Bt corn pollen on monarch butterfly populations: A risk assessment. *Proceedings of the National Academy of Sciences of the United States of America*, 98(21): 11937-11942.

Menassa, R., Nguyen, V., Jevnakir, A., and Brandle, J. (2001). A self-contained system for the field production of plant recombinant interleukin-10. *Molecular Breeding*. 8(2): 177-185.

Miguel, K.S. and Scott, J.G. (2015). The next generation of insecticides: dsRNA is stable as a foliar-appled insecticide. *Pest Management Science*, 72(4): 801-809.

Mikani, A., Wang, Q., and Takeda, M. (2012). Brain-midgut short neuropeptide F mechanism that inhibits digestive activity of the American cockroach, *Periplaneta americana* upon starvation. *Peptides*, 34(1): 135-144.

Miller, S.C., Miyata, K., Brown, S.J., and Tomoyasu, Y. (2012). Dissecting systemic RNA interference in the red flour beetle *Tribolium castaneum*: Parameters affecting the efficiency of RNAi. *PLoS One*, 7(10): e47431.

Miyazaki, S., Iwabuch, K., Pak, J., Fukuhara, T., and Nitta, T. (1996). Selective occurrence of endogenous double-stranded RNAs in insects. *Insect Biochemistry and Molecular Biology*, 8(9): 955-961.

Møldrup, M.E., Geu-Flores, F., de Vos, M., Olsen, C.E., Sun, J., Jander, G., and Halkier, B.A. (2011). Engineering of benzylglucosinolate in tobacco provides proof-of-concept for dead-end trap crops genetically modified to attract *Plutella xylostella* (diamondback moth). *Plant Biotechnology Journal*, 10: 435-442.

Morgenfeld, M., Segretin, M., Wirth, S., Lentz, E., Zelada, A., Mentaberry, A., Gissmann, L., and Bravo-Almonacid, F. (2009). Potato virus X coat protein fusion to human papillomavirus 16 E7 oncoprotein enhance antigen stability and accumulation in tobacco chloroplast. *Molecular Biotechnology*¸ 43(3): 243-249.

Mutti, N.S., Park, Y., Reese, J.C., and Reeck, G.R. (2006). RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*. *Journal of Insect Science*, 6(1): 38.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell*, 2(4): 279-289.

Oerke, E.C. and Dehne, H.W. (2004). Safeguarding production-losses in major crops and the role of crop protection. *Crop Protection*, 23(4): 275-285.

Oey, M., Lohse, M., Kreikemeyer, B., and Bock, R. (2009). Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic. *The Plant Journal*, 57: 436-445.

Panagiotakopuli, E., Buckland, P.C., Day, P.M., and Doumas, C. (1995). Natural insecticides and insect repellents in antiquity: A review of the evidence. *Journal of Archaeological Science*, 22(5): 705-710.

Pardo-López, L., Soberón, M., and Bravo, A. (2012). *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. *FEMS Microbiology Reviews*, 37(1): 3-22.

Pavuk, D. (2009). The tomato hornworm and the tobacco hornworm. *Michigan State University Extension*.

Perlak, F.J., Fuchs, R.L., Dean, D.A., McPherson, S.L., and Fischhoff, D.A. (1991). Modification of the coding sequence enhances plant expression of insect control protein genes. *Proceedings of the National Academy of Science of the United States of America*, 88: 3324-3328.

Petersen, K. and Bock, R. (2011). High-level expression of a suite of thermostable cell wall-degrading enzymes from the chloroplast genome. *Plant Molecular Biology*, 76(3-5): 311-321.

Petrick, J.S., Moore, W.M., Heydens, W.F., Koch, M.S., Sherman, J.H., and Lemke, S.L. (2015). A 28-day oral toxicity evaluation of small interfereing RNAs and a long double-stranded RNA targeting vacuolar ATPase in mice. *Regulatory Toxicology and Pharmacology*, 71(1): 8-23.

Pimentel, D. (2005). Environmental and economic costs of the application of pesticides primarily in the United States. *Environment, Development and Sustainability*, 7: 229-252.

Piñeyro-Nelson, A., van Heerwaarden, J., Perales, H.R., Serratos-Hernandez, J.A., Rangel, A, Hufford, M.B., Gepts, P., Garay-Arroyo, A., Rivera-Bustamante, R., and Alvarez-Buylla, E.R. (2009). Tranegenes in Mexican maize: molecular evidence and methodological considerations for GMO detection in landrace populations. *Molecular Ecology*, 18(4): 750-761.

Porebski, S., Bailey, L.G., and Baum, B.R. (1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Molecular Biology Reporter*, 15(1): 8-15.

Poreddy, S., Li, J., and Baldwin, I.T. (2017). Plant-mediated RNAi silences midgut-expressed genes in congeneric lepidopteran insects in nature. *BMC Plant Biology*, 17: 199.

Porta, H., Jiménez, G., Cordoba, E., León, P., Soberón, M., and Bravo, A. (2011). Tobacco plants expressing the Cry1AbMod toxin suppress tolerance to Cry1Ab toxin of *Manduca sexta* cadherin-silenced larvae. *Insect Biochemistry and Molecular Biology*, 41(7): 513-519.

Prentice, K., Christiaens, O., Pertry, I., Bailey, A., Niblett, C., Ghislain, M., Gheysen, G., and Smagghe, G. (2016). RNAi-based gene silencing through dsRNA injection or ingestion against the African sweet potato weevil *Cylas puncticollis* (Coleoptera: Brentidae). *Pest Management Science*¸ 73(1): 44-52.

Pytelková, J., Hubert, J., Lepšik, M., Šobotnik, J., Šindelka, R., Krízková, I., Horn, M., and Mareš, M. (2009). Digestive α-amylases of the flour moth *Ephestia kuehniella*- adaptation to alkaline environment and plant inhibitors. *The FEBS Journal*, 276(13): 3531-3546.

Quan, G.X., Kanda, T., and Tamura, T. (2002). Induction of the white egg 3 mutant phenotype by injection of double-stranded RNA of the silkworm white gene. *Insect Molecular Biology*, 11: 217-222.

Quist, D., and Chapela, I.H. (2001). Transgenic DNA introgressed into traditional maize landraces in Oaxaca, Mexico. *Nature*, 414: 541-543.

Reinecke, J.P., Bucknerm J.S., and Grugel, S.R. (1980). Life cycle of laboratory-reared tobacco hornworms, *Manduca sexta*, a study of development and behavior, using time-lapse cinematography. *Biological Bulletin,* 158(1): 129-140.

Reisenman, C., Riffell, J., Duffy, K., Pesque, A., Mikles, D., and Goodwin, B. (2013). Species-specific effects of herbivory on the oviposition behavior of the moth *Manduca sexta*. *Journal of Chemical Ecology,* 39(1): 76-89.

Ren, D., Cai, Z., Song, J., Wu, Z., and Zhou, S. (2013). dsRNA uptake and persistence account for tissue-dependent susceptibility to RNA interference in the migratory locust, *Locusta migratoria*. *Insect Molecular Biology,* 23(2): 175-184.

Ren, N. and Timko, M.P. (2001). AFLP analysis of genetic polymorphism and evolutionary relationships among cultivated and wild Nicotiana species. *Genome,* 44(4): 559-571.

Rowan, B.A., Oldenburg, D.J., and Bendich, A.J. (2010). RecA maintains the integrity of chloroplast DNA molecules in Arabidopsis. *Journal of Experimental Botany,* 61(10): 2575-2588.

Rowley, M. and Hanson, F. (2007). Humidity detection and hygropreference behavior in larvae of the tobacco hornworm, *Manduca sexta*. *Journal of Insect Science,* 7(39): 1-10.

Saleh, M., van Rij, R.P., Hekele, A., Gillis, A., Foley, E., and O'Farrell, P.H. (2006). The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. *Nature Cell Biology*, 8: 793-802.

Sanscrainte, N.D., Arimoto, H., Waits, C.M., Li, L.Y., and Johnson, D. (2018). Reduction in *Musca domestica* fecundity by dsRNA-mediated gene knockdown. *PLoS One*, 13(1): e0187353.

Santos, V.C., de Sigueira, H.A., da Silva, J.E., and de Farias, M.J. (2011). Insecticide resistance in populations of the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), from the state of Pernambuco, Brazil. *Neotropical Entomology,* 40(2): 264-270.

Sasaki, M. and Riddiford, L.M. (1984). Regulation of reproductive behavior and egg maturation in the tobacco hawk moth, *Manduca sexta*. *Physiological Entomology*, 9: 315-327.

Saxena, S., Jónsson, Z.O., and Dutta, A. (2003). Small RNAs with imperfect match to endogenous mRNA repress translation: Implications for off-target activity of small inhibitory RNA in mammalian cells. *The Journal of Biological Chemistry*, 278: 44312-44319.

Schernthaner, J.P., Milne, R.E., and Kaplan, H. (2002). Characterization of a novel insect digestive DNase with a highly alkaline pH optimum. *Insect Biochemistry and Molecular Biology,* 32(3): 255-263.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., and Dean, D.H. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews,* 62(3): 775-806.

Schoel, B. and Fagan, J. (2009). Insufficient evidence for the discovery of transgenes in Mexican landraces. *Molecular Ecology,* 18: 4143-4144.

Schroeder, M.E. and Flattum, R.F. (1984). The mode of action and neurotoxic properties of the nitromethylene heterocycle insecticides. *Pesticide Biochemistry and Physiology,* 22(2): 148-160.

Schwarz, D.S., Hutvágner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P.D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. *Cell*, 115(2): 199-208.

Sears, M.K., Hellmich, R.L., Stanley-Horn, D.E., Mattila, H.R., Siegfried, B.D., and Dively, G.P. (2001). Impact of Bt pollen on monarch butterfly populations: a risk assessment. *Proceedings of the National Academy of Sciences of the United States of America,* 98(21): 11937-11942.

Sharma, N., Tripathi, A., and Sanan-Mishra, N. (2015). Profiling the expression domains of a rice-specific microRNA under stress. *Frontiers in Plant Science,* 6: 1-12.

Shelton, A.M., Zhao, J.Z., and Roush, R.T. (2002). Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. *Annual Review of Entomology*, 47(1): 845-881.

Shih, J.D., Fitzgerald, M.C., Sutherlin, M., and Hunter, C.P. (2009). The SID-1 double-stranded RNA transporter is not selective for dsRNA length. *RNA*, 15(3): 384-390.

Shih, J.D. and Hunter, C.P. (2011). SID-1 is a dsRNA-selective dsRNA-gated channel. *RNA*, 17(6): 1057-1065.

Shukla, J.N., Kalsi, M., Sethi, A., Narva, K.E., Fishilevich, E., Singh, S., Mogilicherla, K., and Palli, S.R. (2016). Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects. *RNA Biology*, 13(7): 656-669.

Sierro, N., Battey, J.N.D., Ouadi, S., Bovet, L., Goepfert, S., Bakaher, N., Peitsch, M.C., and Ivanov, N.V. (2013). Reference genomes and transcriptomes of *Nicotiana sylvestris* and *Nicotiana tomentosiformis*. *Genome Biology*, 14: R60.

Southern, E. (2006). Southern blotting. *Nature Protocols*, 1(2): 518-525.

Staub, J.M., Garcia, B., Graves, J., Hajdukiewicz, P.T.J., Hunter, P., Nehra, V., Schlittler, M., Carroll, J.A., Spatola, L., Ward, D., Ye, G., and Russell, D.A. (2000). High-yield production of a human therapeutic protein in tobacco chloroplasts. *Nature Biotechnology*, 18: 333-338.

Svab, Z., Hajdukiewicz, P., and Maliga, P. (1990). Stable transformation of plastids in higher plants. *Proceedings of the National Academy of Sciences of the United States of America*, 87(21): 8526-8530.

Svab, Z., Harper, E.C., Jones, J.D., and Maliga, P. (1990). Aminoglycoside-3”-adenyltransferase confers resistance to spectinomycin and streptomycin in Nicotiana tabacum. *Plant Molecular Biology*, 14(2): 197-205.

Svab, Z. and Maliga, P. (1993). High-frequency plastid transformation in tobacco by selection for a chimeric aadA gene. *Proceedings of the National Academy of Sciences of the United States of America*, 90: 913-917.

Tian, H., Han, P., Yao, Q., Chen, H., Xie, Q., Tang, B., and Zhang, W. (2009). Developmental control of a lepidopteran pest *Spodoptera exigua* by ingestion of bacteria expressing dsRNA of a non-midgut gene. *PLoS One*, 4(7): e6225.

Tomoyasu, Y., Miller, S.C., Tomita, S., Schoppmeier, M., Grossmann, D., and Bucher, G. (2008). Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in Tribolium. *Genome Biology*, 9(1): R10.

Tomoyasu, Y. and Denell, R.E. (2004). Larval RNAi in Tribolium (Coleoptera) for analyzing adult development. *Developmental Genes and Evolution*, 214: 575-578.

Turner, C.T., Davy, M.W., MacDiarmid, M., Plummer, K.M., Birch, N.P., and Newcomb, R.D. (2006). RNA interference in the light brown apple moth, *Epiphyas postvittana* (Walker) induced by double-stranded RNA feeding. *Insect Molecular Biology*, 15(3): 383-391.

Ulvila, J., Parikka, M., Kleino, A., Sormunen, R., Ezekowitz, R.A., Kocks, C., and Rämet, M. (2006). Double-stranded RNA is internalized by scavenger receptor-mediated endocytosis in Drosophila S2 cells. *The Journal of Biological Chemistry*, 281(20): 14370-14375.

Vaeck, M., Reynaerts, A., Höfte, H., Jansens, S., De Beuckeleer, M., Dean, C., Zabeau, M., Van Montagu, M., and Leemans, J. (1987). Transgenic plants protected from insect attack. *Nature*, 328: 33-38.

Verma, D., Samson, N.P., Koya, V., and Daniell, H. (2008). A protocol for expression of foreign genes in chloroplasts. *Nature Protocols*, 3(4): 739-758.

Wang, K., Peng, Y., Pu, J., Fu, W., Wang, J., and Hun, Z. (2016). Variation in RNAi efficacy among insect species is attributable to dsRNA degradation in vivo. *Insect Biochemistry and Molecular Biology*, 77: 1-9.

Wang, M., Weiberg, A., Lin, F, Thomma, B. P. H. J., Huang, H., and Jin, H. (2016). Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nature Plants*, 2: 1-10.

Wang, Y., Zhang, H., Li, H., and Miao, X. (2011). Second-generation sequencing supplies an effective way to screen RNAi targets in large scale for potential application in pest insect control. *PLoS One*, 6(4): e18644.

Wieczorek, H., Beyenback, K.W., Huss, M., and Vitavska, O. (2009). Vacuolar-type proton pumps in insect epithelia. *Journal of Experimental Biology*, 212(11): 1611-1619.

Whyard, S., Singh, A.D., and Wong, S. (2009). Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochemistry and Molecular Biology*, 39(11): 824-832.

Winston, W.M., Molodowitch, C., and Hunter, C.P. (2002). Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. *Science*, 295(5564): 2456-2459.

Winston, W.M., Sutherlin, M., Wright, A.J., Feinberg, E.H., and Hunter, C.P. (2007). *Caenorhabditis elegans* SID-2 is required for environmental RNA interference. *Proceedings of the National Academy of the Sciences of the United States of America*, 104(25): 10565-10570.

Wood, T.J. and Goulson, D. (2017). The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013. *Environmental Science and Pollution Research*, 24(21): 17285-17325.

Woodcock, B.A., Ridding, L., Freeman, S.N., Pereira, M.G., Sleep, D., Redhead, J., Aston, D., Carreck, N.L., Shore, R.F., Bullock, J.M., Heard, M.S., and Pywell, R.F. (2018). Neonicotinoid residues in UK honey despite European Union moratorium. *PLoS One*, 13(1): e0189681.

Wuriyanghan, H., Rosa, C., and Falk, B.W. (2011). Oral delivery of double-stranded RNAs and siRNAs induces RNAi effects in the potato/tomato psyllid, Bactericerca cockerelli. *PLoS One*, 6(11): e27736.

Xiang, S., Fruehauf, J., and Li, C.J. (2006). Short hairpin RNA-expressing bacteria elicit RNA interference in mammals. *Nature Biotechnology*, 24(6): 697-701.

Xu, W. and Han, Z. (2008). Cloning and phylogenetic analysis of sid-1-like genes from aphids. *Journal of Insect Science*, 8: 1-6.

Yamamoto, R.T. (1969). Mass rearing of the tobacco hornworm. II. Larval rearing and pupation. *Journal of Economic Entomology*, 62(6): 1427-1431.

Yang, J. and Han, Z. (2014). Efficiency of different methods for dsRNA delivery in cotton bollworm (*Helicoverpa armigera*). *Journal of Integrative Agriculture*, 13(1): 115-123.

Yoon, J.S., Gurusamy, D., and Palli, S.R. (2017). Accumulation of dsRNA in endosomes contributes to inefficient RNA interference in the fall armyworm, *Spodoptera frugiperda*. *Insect Biochemistry and Molecular Biology*, 90: 53-60.

Yu, N., Christiaens, O., Liu, J., Niu, J., Cappelle, K., Caccia, S., Huvenne, H., and Smagghe, G. (2013). Delivery of dsRNA for RNAi in insects; an overview and future directions. *Insect Science*, 20: 4-14.

Yu, X., Gowda, S., and Killiny, N. (2017). Double-stranded RNA delivery through soaking mediates silencing of the muscle protein 20 and increases mortality to the Asian citrus psyllid, *Diaphornia citri*. *Pest Management Science*, 73(9): 1846-1853.

Zhang, J., Khan, S.A., Hasse, C., Ruf, S., Heckel, D.G., and Bock, R. (2015). Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids. *Science*¸ 3479(6225): 991-994.

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